

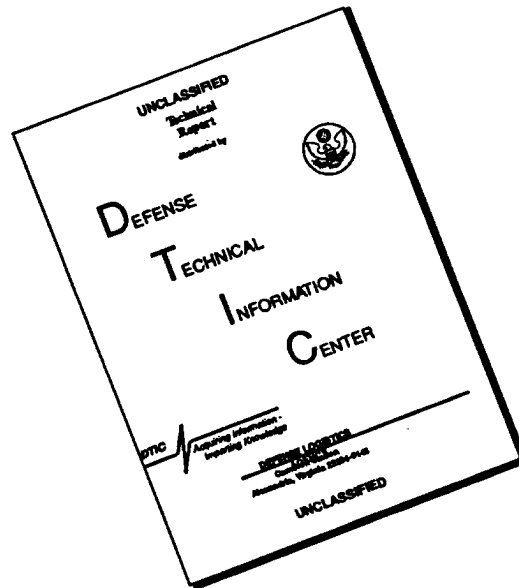
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<p>The overall objective of this research project was to explore the use of in situ bio-monitoring using wild mammalian animal models to assess ecotoxicity risks from petrochemical contaminants. We approached this objective by comparing the relative sensitivities of selected measures of metabolic, immunologic, genetic, and histopathologic toxicity (multi-parameter model) in small-mammalian residents of terrestrial ecosystems contaminated with complex mixtures of petrochemicals (an abandoned oil refinery complex). Multiparameter response profiles of small mammals were evaluated relative to results from common laboratory bioassay tests (fathead minnow survival, rice seed germination test, etc.) and soil chemical analyses to determine their ability to predict ecotoxicity risks (as indexed by demographic changes in the small mammal community). Our principal in situ biomonitor was the cotton rat (<i>Sigmodon hispidus</i>), which is the dominant member of the small mammal community on 3 uncontaminated reference and 3 heavy metal-petrochemical contaminated study sites. Chemical analyses of soil and soil extracts identified a variety of heavy metal and organic contaminants.</p> <p>(Over)</p>							
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nants on the 3 suspected toxic study sites, which was reflected in common laboratory bioassay results using fathead minnow, microtox, rice seed germination, and Ceriodaphnia assays. At the ecosystem level, in situ small mammal total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity; community profiles included measurements of diversity, richness, and similarity. Both population and community indices demonstrated sensitivity as ecosystem endpoint markers of contaminant exposure, with density and survival estimates differing significantly between toxic and reference study sites. Altered immune function (differences in measures of cell-mediated immunity, circulating levels of immune cells, and immune organ development) was detected, which could have increased their susceptibility to infection and disease and may have contributed to the observed increased juvenile mortality on toxic sites. Pathological examinations proved very useful in assessing toxicity, with dental lesions attributed to fluoride toxicity being evident in most animals collected from petrochemical-contaminated sites. Analysis of methoxyresorufin (MROD) and ethoxyresorufin (EROD) O-dealkylase activity of cytochrome P-450 enzyme systems also indicated the presence of contaminant-induced lesions. Chromosome aberration and flow cytometric analyses suggests that animals were suffering induced chromosome lesions on toxic study sites. Seasonal effects in the degree and type of physiologic lesions documented in resident small mammals were observed for metabolic, cytogenetic, and immunologic end-points, suggesting seasonal changes may have occurred in the volatility of organic contaminants on toxic study sites. Validation of our proposed in situ mammalian multiparameter model should be undertaken.

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**FINAL TECHNICAL REPORT**

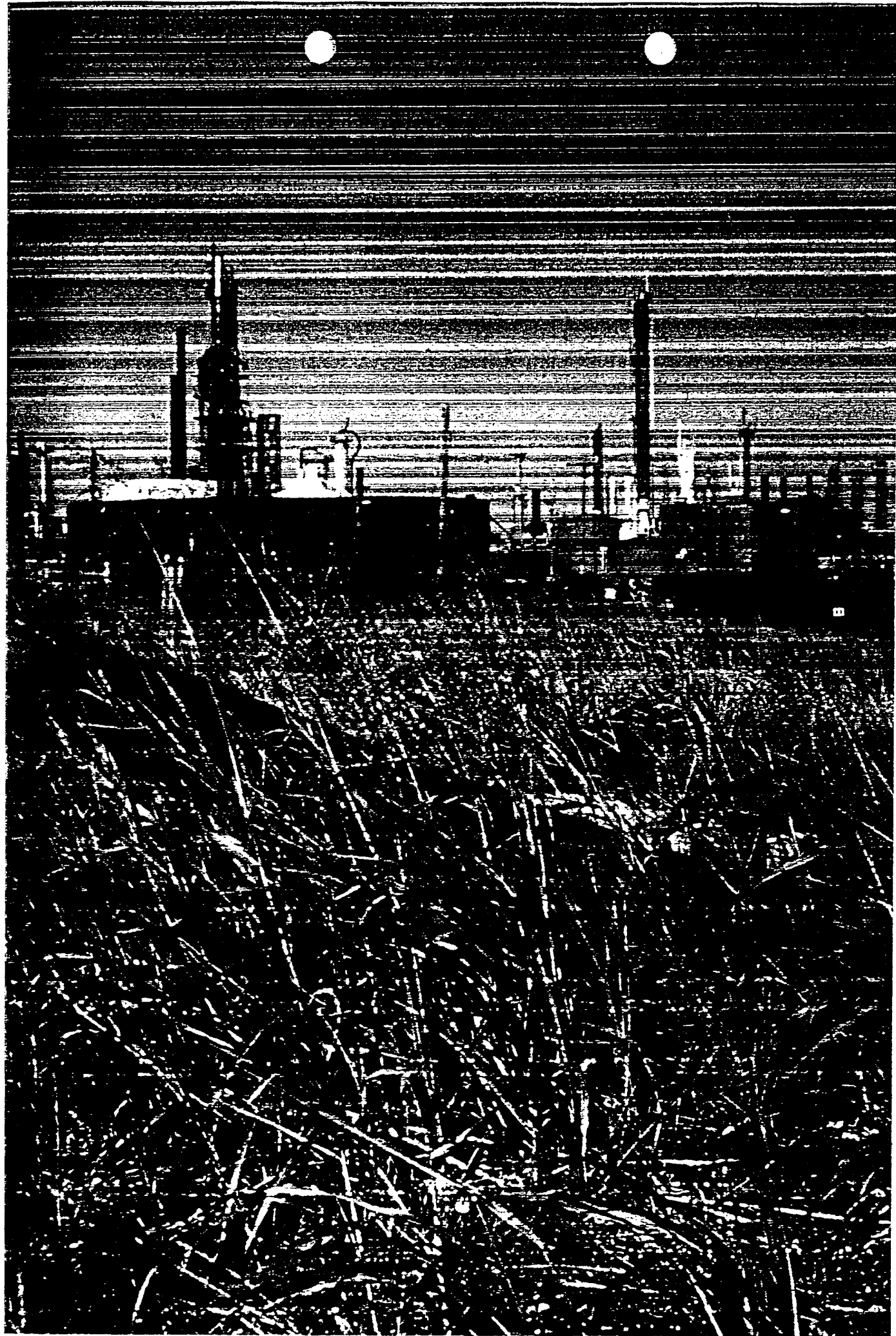
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**Wild Mammalian Biomonitors for Assessing Impacts of  
Environmental Contamination on  
Population and Community Ecology**

31 October 1994

Prepared by  
Environmental Toxicology Program  
Department of Zoology  
Oklahoma State University

19950613 104



## FINAL TECHNICAL REPORT

### Wild Mammalian Biomonitoring for Assessing Impacts of Environmental Contamination on Population and Community Ecology.

#### EXECUTIVE SUMMARY

The overall objective of this research project was to explore the use of in situ biomonitoring using wild mammalian animal models to assess ecotoxicity risks from petrochemical contaminants. We approached this objective by comparing the relative sensitivities of selected measures of metabolic, immunologic, genetic, and histopathologic toxicity (multiparameter model) in small-mammalian residents of terrestrial ecosystems contaminated with complex mixtures of petrochemicals (an abandoned oil refinery complex). Multiparameter response profiles of small mammals were evaluated relative to results from common laboratory bioassay tests (fathead minnow survival, rice seed germination test, etc.) and soil chemical analyses to determine their ability to predict ecotoxicity risks (as indexed by demographic changes in the small mammal community). Our principal in situ biomonitor was the cotton rat (*Sigmodon hispidus*), which is the dominant member of the small mammal community on 3 uncontaminated reference and 3 heavy metal-petrochemical contaminated study sites. Chemical analyses of soil and soil extracts identified a variety of heavy metal and organic contaminants on the 3 suspected toxic study sites, which was reflected in common laboratory bioassay results using fathead minnow, microtox, rice seed germination, and *Ceriodaphnia* assays. At the ecosystem level, in situ small mammal total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity; community profiles included measurements of diversity, richness, and similarity. Both population and community indices demonstrated sensitivity as ecosystem endpoint markers of contaminant exposure, with density and survival estimates differing significantly between toxic and reference study sites. Altered immune function (differences in measures of cell-mediated immunity, circulating levels of immune cells, and immune organ development) was detected, which could have increased their susceptibility to infection and disease and may have contributed to the observed increased juvenile mortality on toxic sites. Pathological examinations proved very useful in assessing toxicity, with dental lesions attributed to fluoride toxicity being evident in most animals collected from petrochemical-contaminated sites. Analysis of methoxyresorufin (MROD) and ethoxyresorufin (EROD) O-dealkylase activity of cytochrome P-450 enzyme systems also indicated the presence of contaminant-induced lesions. Chromosome aberration and flow cytometric analyses suggest that animals were suffering induced chromosome lesions on toxic study sites. Seasonal effects in the degree and type of physiologic lesions documented in resident small mammals were observed for metabolic, cytogenetic, and immunologic endpoints, suggesting seasonal changes may have occurred in the volatility of organic contaminants on toxic study sites. Validation of our proposed in situ mammalian multiparameter model should be undertaken.

## REPORT PERIOD

01 Jun 91- 31 Aug 94

## PRINCIPAL INVESTIGATORS:

Dr. Robert L. Lochmiller  
 Dr. Karen McBee  
 Dr. Charles A. Qualls, Jr.  
 Dr. S. L. (Bud) Burks

## OTHER PROJECT PERSONNEL

Several graduate students and laboratory technicians were both directly and indirectly supported by this research grant, or they provided valuable technical assistance to this project. Additionally, we obtain the advice of, used the laboratory facilities of, or collaborated with several professionals on this USAF-sponsored research project. The following comprehensive list details the many individuals that contributed to the overall three-year project:

### Graduate Student/technicians

Hamid R. Amouzadeh (Ph.D. candidate- now Postdoctoral candidate at Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, Bethesda MD)  
 Chandikumar S. Elangbam (Ph.D. candidate- now research pathologist at Will Research Institute, Ashland OH)  
 Sundeep Chandra (Ph.D. candidate)  
 Chun Lin Chen (Ph.D. candidate- now Postdoctoral candidate at Saint Jude Hospital, Memphis TN)  
 Barbara C. Bowers (M.S. candidate)  
 Susan L. Hedinger (M.S. candidate)  
 Soochong Kim (M.S. candidate. Veterinary Pathology)  
 Jim Lish (Laboratory technician)  
 Corinne McMillan (M.S. candidate)  
 Scott T. McMurry (Ph.D candidate- now asst. prof. at Clemson U.)  
 Madav Parangipe (Postdoc candidate- now assist. prof. La. State U.)  
 Timothy Propst (M.S. candidate)  
 Ananda Ramanathan (Ph.D. candidate)  
 Joseph Roder (Ph.D. candidate)  
 Andrea Sampley (Laboratory technician)  
 Eric L. Stair (M.S. candidate, Veterinary Pathology)  
 Kathleen Thies (Research Technician)  
 Monte L. Thies (Ph.D. candidate- now asst. prof. at SamHouston State Univ. TX)  
 Garry Yates (M.S. candidate- now Ph.D. candidate in environ. engineer.)  
 Jing Ren Zhang (Ph.D. candidate- now in Med. Sch. at U. Houston)  
 Cathy Butchko (Research Technician)  
 Susan Hedinger (M.S. candidate)

### Professional Collaborators on Project

Dr. Paul McCay, Oklahoma Medical Research Foundation , gave advise and assayed samples for free radicals.

Dr. Michael Rohrer, University of Oklahoma, College of Dentistry, processed undecalcified sections of rat teeth and assisted in histologic interpretation of dental lesions.

Dr. Gunda Reddy, United States Army Environmental Hygiene Agency, Fort Detrick MD, gave advise on metabolic assays

Dr. Ronald Tyrl, Oklahoma State Univ. Dept. Botany, provided expertise in identification of plants on study sites.

Dr. William Warde, Oklahoma State Univ. Dept. Statistics, provided statistical expertise to the project.

Dr. Jim Ownby, Oklahoma State Univ. Dept. of Botany, provided valuable guidance and access to specialized equipment for phytotoxicity assays and analyses of "stress" proteins.

Dr. David Waits, Oklahoma State Univ. Geography Dept., provided guidance for building Geographic Information System (GIS) data layers to allow us to visually portray the spatial relationships of the chemical contaminant and toxic effects data.

## RESEARCH OBJECTIVES

### Objective I

To determine the ecological effects and sensitivity of complex mixtures of environmental toxicants at contaminated petrochemical waste sites on the structure and composition of resident populations and communities of small mammals by quantitating:

1. intrinsic attributes of populations (age structure, sex ratio, recruitment rate, survival rate, density, population turnover rate).
2. community level measures of integrity (species richness, diversity, similarity of communities).

### Objective II

To determine the immunotoxicity, genotoxicity, and metabolic toxicity (physiological response-profiles) of complex mixtures of environmental toxicants in resident small mammals inhabiting contaminated petrochemical waste sites through evaluation of:

1. immune organ development, lymphocyte subtyping, lymphoproliferation, humoral and cell-mediated immunity.
2. chromosome aberrations, variation in nuclear DNA content, DNA strandbreaks.
3. total and isoenzyme levels of hepatic cytochrome P-450, ultrastructural pathology, free radical concentration.

### Objective III

To evaluate the use of enclosed terrestrial mesocosms for conducting both subacute and chronic in situ exposures of cotton rats to complex mixtures of environmental toxicants at contaminated petrochemical waste sites by measuring:

1. reproductive and survival response.
2. physiological response-profiles.



## Objective IV

To compare in situ physiological response-profiles (immunologic, genetic, metabolic endpoints) of wild mammalian biomonitors to a variety of standard laboratory-derived biological assays for soil leachates from contaminated petrochemical waste sites by using:

1. *Ceriodaphnia* short-term chronic bioassay.
2. fathead minnow larval survival bioassay.
3. rice seed germination toxicity assay.
4. Microtox

## RESEARCH OVERVIEW

### Study Site

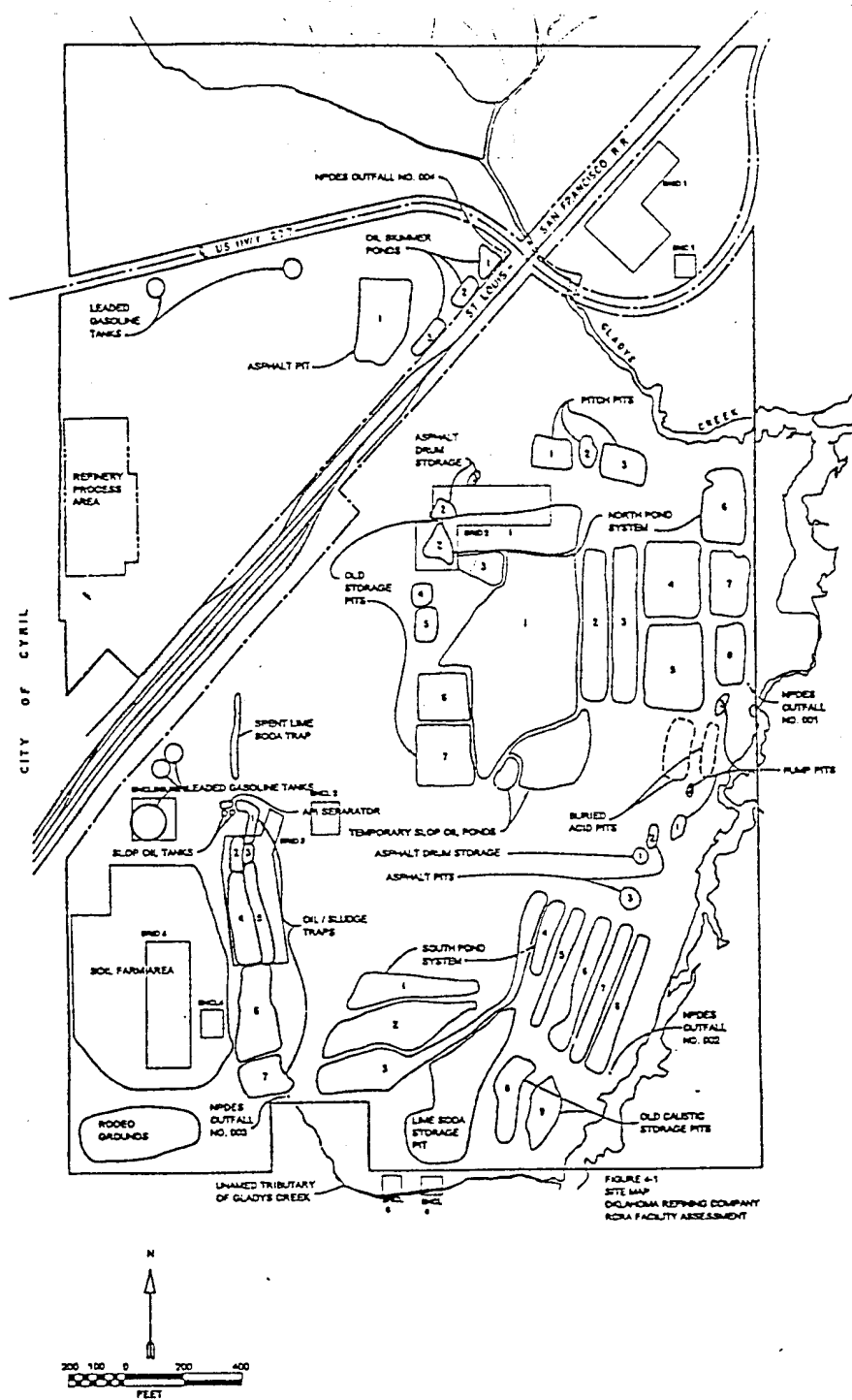
We selected six areas for intensive study, located on or adjacent to a large 160 acre oil refinery complex that has been a declared Superfund Waste Site for several years (Oklahoma Oil Refinery Superfund Site, Cyril, Oklahoma). This site was selected for study because of its large size as well as the variety of petrochemical contaminants found on site. The refinery operated from 1920 to 1984, during which process wastes were deposited in over 50 different impoundments and applied to the soil in a landfarming operation. Earlier EPA reports indicated the area was heavily contaminated with petroleum related organic compounds (benzene, ethylbenzene, toluene, naphthalene, phenols, etc.), metals (lead, chromium, arsenic, etc.), and caustic wastes. We used these earlier surveys to select specific areas for detailed biomonitoring. A prerequisite was the occurrence of a viable small mammal community and similarity in vegetation structure and composition among areas (to control for possible nutritional effects, predation, and other factors). All six study sites (3 reference, 3 suspected petrochemical-contaminated study sites) represented classic disturbed ecosystems that were vegetatively dominated by Johnson grass and a variety of forbs. The three contaminated sites include a sludge land-farming area containing heavy metal and petroleum contaminants, a spoil-site adjacent to a series of API-separator ponds containing heavy metal and petroleum contaminants, and a site adjacent to asphalt disposal pits containing a variety of petroleum contaminants (Fig. 1). Additionally, we selected six smaller sites on the refinery to construct mesocosms for short-term in situ exposures of small mammals.

We established permanent population monitoring grids at each of the six locations. Small mammal communities were censused seasonally for 2.5 years and individual animals returned to the laboratory for profiling metabolic, genotoxic, immunotoxic, and histopathologic responses (both resident and mesocosm-introduced animals) on the Oklahoma Oil Refinery Superfund Site, Cyril, Oklahoma.

### Contaminants and Bioassay Results

A primary objective of our overall research efforts was to evaluate correlations between laboratory derived toxicity response factors of aquatic invertebrates, fish, and plants with community response factors of resident small mammals living in ecosystems contaminated with petrochemical hazardous wastes. As a secondary objective, we compared chemical contaminant concentration data from soil samples removed from the study site areas with EPA recommended Chronic Toxicity Reference (CTR) Levels specified in the Toxics Characteristics (TC) rules (EPA, March 29, 1990, Fed. Reg., 55(61)11796-11877). EPA established the chronic toxicity reference levels on the basis of potential human health effects from consuming groundwater contaminated with concentrations greater than the CTR levels; thus, the question of ecosystem effects was not considered in establishing TC regulatory levels. EPA clearly recognized the potential that CTR levels based on human health effects might not protect the ecosystem and

Fig. 1. Site location map illustrating position of trap grids and mesocosms on the Oklahoma Refining Co. Superfund Waste Site, Cyril, OK.



stated; "When sufficient information concerning these ecological risks is available, the Agency will compare the ecological-risk-based levels to the TC regulatory levels to determine whether further revisions to the levels, based on ecological risk are necessary." Data collected as a result of our research efforts provides much of the missing data on ecological risks.

We evaluated the use of the EPA Toxic Characteristics Leachate Procedure (TCLP) for extraction of soil samples from the Cyril Refinery. The original proposal was to use the TCLP to provide aqueous extracts which could be analyzed for chemical contaminants as well as performing toxicity tests with aquatic organisms. The TCLP procedure did not provide a good protocol for obtaining samples for toxicity tests with aquatic organisms, since the blank caused too much toxicity to be acceptable. We returned to using reconstituted water as the extracting fluid (U.S. Army Corps of Engineers 1991). The aqueous extraction procedure imparted no solvent blank toxicity, but it may not simulate "worst-case" conditions that could occur in the field situations.

### Chemical Contaminants

The concentration of toxic metals in soil samples collected from study sites (Grids 1 through 6 and Mesocosms 1 through 6) on the Refinery Superfund Waste Site indicated relatively high concentrations of cadmium, zinc, manganese, copper, lead, sodium, chloride, and sulfates. Among these, Mesocosm 3 (adjacent to API oil separator ponds) soil extracts exhibited highest concentration of potassium, arsenic, chromium, sodium, chloride, sulfate, and COD of all contaminated sites (Table 1). Small mammal population monitoring site adjacent to the same API oil separator ponds (Grid 3) exhibited the highest concentrations of manganese and iron. Grid 4 (land farm for waste oils) contained the highest observed levels of zinc, cadmium, and lead; whereas, Mesocosm 4 contained the highest levels of nitrates. The highest concentration of fluorides (141 ng/g) occurred on one of our reference Mesocosms (Mesocosm 5), which was located just outside the old refinery property boundary. We never observed evidence of fluorosis in animals removed from this Mesocosm however. Fluorides were also extremely high on Grids 3 and 4, often with concentrations > 100 ng/g of soil.

The level of chemical oxygen demand (COD) in the TCLP extract of Mesocosm 3 soil was >10 fold higher than the next highest sample. The COD, a good index of organic contaminant concentrations in soil, suggested that Mesocosm 3 soils were highly contaminated with organic chemicals. HPLC and Gas Chromatographic analyses of the TCLP extracts from Mesocosm 3 indicated a complex mixture of hydrocarbons with chromatographic retention times between that of trimethyl benzene to ethyl naphthalene. Both the TCLP and the aqueous extracts from Mesocosm 3 were dark brown in color in contrast to colorless extracts from the other sites. The color was not removed by filtration with 0.45 micrometer cellulose membrane filter, centrifugation at 10,000 rpm, and passage through activated carbon.

### Aquatic Toxicity Test Results.

The three contaminated enclosures showed differential responses among the organisms tested (Tables 2 and 3). Aqueous soil leachates from Mesocosm 3 were highly lethal to *Ceriodaphnia* and fathead minnows; and rice seeds did not germinate when tested in Mesocosm 3 soil. The soil from this study site had a noxious odor and appeared to be saturated with oily contaminants, making it difficult to extract. The aqueous extract was dark brown in color, turbid and had an oily appearance and odor. Aqueous leachates of Mesocosm 3 soil collected in 1992 caused 100% mortality of *Ceriodaphnia* exposed to 0.01% soil extract. The threshold of lethal effects was very sharp since at 0.005% leachate there was 100% survival. An LC50 could not be calculated by probit method, since there were no intermediate responses between 100% and 0% mortality. The 1993 Mesocosm 3 soil was comparatively less toxic than 1992 samples and *Ceriodaphnia* survival LC50 value had increased to 12.85%. But sublethal effects still persisted at low concentration as seen in partial chronic test and a significant effect upon *Ceriodaphnia* neonate production was observed in 0.01% extract. Mesocosm 3 soil collected in 1992 also

**Table 1** Synopsis of Chemical Analyses of TCLP Extracts of Soil and Soil Samples from Cyril Refinery, 1991-93.

Cyril Data		1993		Metals		ng/g of soil		extracted		Cd		Pb		As		Na		Cr		Anions		Cl		NO2		NO3		PO4		SO4		Organic		COD (mg/l)		COD Tnt		MTox NOEC % dilutio		MTox EC 50 % dilutio		48hour, 100 Cero % mort				
Sample Date	Analysis Date	Site #	K	(log/g)	Cu	Zn	Mn	Fe		Cd	Pb	As	Na	Cr		FI																														
Dec-91	Jan-92	Grid1	1.9	3.5	84.0	1207.4	0.06	2.13																																						
Dec-91	Jan-92	Grid2	52.6	6.14	1049.0	0.07	5.17																																							
Dec-91	Jan-92	Grid3	419.8	6.5	200	150.4	3410.0	0.10	11.20																																					
Dec-91	Jan-92	Grid4	423.4	2.9	4.9	110.4	2064.2	0.06	4.87																																					
Dec-91	Jan-92	Grid5	543.4	25.9	49.3	117.9	2133.0	0.18	79.15																																					
Jan-92	Apr-92	Grid2	217.5	1.9	2.9	3.5	6.7	0.02	0.12																																					
Jan-92	Apr-92	Grid3	193.1	0.2	4.3	5.2	12.2	0.02	0.33																																					
Jan-92	Apr-92	Grid4	209.4	0.5	1.6	4.3	20.6	0.02	2.13																																					
Jan-92	Apr-92	Grid5	192.3	3.2	1.5	1.8	11.3	0.00	0.06																																					
Jan-92	Apr-92	Grid6	120.0	1.3	0.5	8.1	73.1	0.01	0.22																																					
Jan-92	May-92	Grid1	294.2	10.3	0.4	0.5	40.0	0.01	0.24																																					
Jan-92	May-92	Grid2	85.0	9.2	0.2	30.2	37.0	0.00	0.04																																					
Jan-92	May-92	Grid3	113.4	26.4	0.2	18.6	17.4	0.01	16.00																																					
Jan-92	May-92	Grid4	400.4	155.0	0.6	11.5	59.0	0.01	10.92																																					
Jan-92	Jun-92	Grid1	88.2	0.1	0.5	0.6	151.9																																							
Jan-92	Jun-92	Grid2	55.8	0.2	0.2	0.3	9.9	0.00	0.10	0.05																																				
Jan-92	Jun-92	Grid3	73.7	0.4	0.2	0.4	12.4	0.00	0.26	0.06																																				
Jan-92	Jun-92	Grid4	76.0	0.1	0.5	0.5	192.2	0.00	0.29	1.49																																				
Jan-92	Jun-92	Grid5	874.6	1.6	13.3	4.2	2601.2	0.01	56.03	10.78																																				
Jan-93	Feb-93	Grid1	51.7	0.1	0.1	3.5	0.00	0.11	0.04	6.9	0.11																																			
Jan-93	Feb-93	Grid2																																												
Jan-93	Feb-93	Grid3																																												
Jan-93	Feb-93	Grid4																																												
Jan-93	Feb-93	Grid5																																												
Jan-93	Feb-93	Grid6																																												
Encl1 avg	SD		185.1	5.2	0.4	0.5	116.1	0.004	0.264	0.894			3.9	0.320		1.67																														
Encl 2 avg	SD		154.3	7.3	0.1	0.0	107.6	0.003	0.033	0.840			ERR	ERR		2.66																														
Encl 3 avg	SD		85.0	9.2	0.2	30.2	37.0	0.004	0.040	0.120						4.36																														
Encl 4 avg	SD		470.4	10.3	6.1	44.4	1560.9	0.025	25.632	5.450			2284.5	3.934		39.61																														
Encl 5 avg	SD		382.8	14.0	6.6	57.6	1363.4	0.030	26.905	7.538			ERR	ERR		23.33																														
Encl 6 avg	SD		226.1	7.7	1.3	5.8	31.2	0.015	5.515	0.041			6.9	0.171		32.86																														
Encl 7 avg	SD		246.5	109.5	1.8	8.0	39.3	0.021	7.644	0.001			ERR	ERR		4.67																														
Encl 8 avg	SD		192.3	3.2	1.5	1.8	11.3	0.002	0.060							91.45																														
Encl 9 avg	SD		120.0	1.3	0.5	8.1	73.1	0.010	0.217							2.05																														
Encl 10 avg	SD		270.2	1.0	2.0	42.3	719.7	0.030	1.190				2.5	0.284		0.00																														
Encl 11 avg	SD		257.4	1.3	2.2	59.0	802.9	0.040	1.324				ERR	ERR		ERR																														
Encl 12 avg	SD		372.0	5.6	20.7	32.5	927.9	0.049	2.646							44.20																														
Encl 13 avg	SD		218.5	5.2	24.7	40.9	737.0	0.036	3.576							ERR																														
Encl 14 avg	SD		222.9	2.3	8.2	52.0	1144.0	0.041	3.876	0.051			9.1	0.472		72.71																														
Encl 15 avg	SD		183.8	3.6	10.4	85.2	1962.4	0.052	6.344	ERR			ERR	ERR		47.65																														
Encl 16 avg	SD		241.7	9.2	17.0	40.8	722.2	0.055	27.616	0.059			14.7	0.153		79.60																														
Encl 17 avg	SD		215.7	15.3	28.0	66.7	1222.4	0.058	44.633	ERR			ERR	ERR		ERR																														

caused significant toxic effect to fathead minnow larval survival and 100% mortality was observed in 0.1% soil extract which was 10 fold higher value than *Ceriodaphnia* survival. The NOEC (no observed effect concentration) value for fish survival was 0.01%. But no significant effect upon fish larval growth was observed. The effects of Mesocosm 3 soil samples collected in 1993 upon *Ceriodaphnia* were similar to 1992, i.e., exhibited low toxicity to fish. Fish survival was reduced to 25% in 20% soil extract. Toxicity tests with daphnids and fish larvae could not be conducted above 20% soil extract, since the dark color of the extract prevented visual determination of toxic effects upon daphnid neonates and fish larvae.

Soil from Mesocosm 2 (old leaded gasoline tank battery site), while not as acutely toxic as Mesocosm 3 soil, had significant effects on organisms tested as well. Soil collected in 1992 had no toxic effect on survival but suppressed 78% *Ceriodaphnia* reproduction (Table 2). In comparison, soil collected in 1993 caused 100% mortality in 100% soil extract and 0% *Ceriodaphnia* reproduction in 50% soil extract. The abnormality such as shedding the eggs by the adult was observed in 50% soil extract. The Mesocosm 2 soil extract (100%) had no significant effect on fathead minnow larval survival and growth.

### Phytotoxicity Indices

Initially the seed germination test was conducted for five days but was later extended up to 10 days to determine the maximum germination rates. We observed that consecutively in 1992 and 1993 seed germination was 0% in Mesocosm 3 soil on day 5, but it increased above 50% on day 10. Phytotoxic effects persisted in two years which could be due to seeds having direct contact with similar soil pollutants. Though 100% soil showed around 50% germination, the next dilution namely 50% showed no significant toxic effect.

In this study we found that shoot dry weight was a better measure of phytotoxicity than shoot length. Plants grown in reference soils were more robust and healthier, with greater shoot weight compared to those grown in the contaminated soils. Mesocosm 3 soil collected in 1992 and 1993 suppressed shoot dry weight 81% and 71% when compared to length of plants grown in soils from Grid 1 (reference site). Additionally, morphological abnormalities in root growth were also observed, including stubby and thick roots. It was interesting to note that observations that Mesocosm 3 soil collected in 1993 were less toxic to rice root growth than the previous year were in agreement with results from aquatic animal toxicity tests described above. In this study both root length and root dry weight were judged to be useful measures to determine toxic effects of the oil refinery wastes.

We found that soil from Grids 3 (adjacent to API oil separator ponds) and 4 (land farm for waste oils) and Mesocosm 2 (old leaded gas tank battery site) never showed any toxic effect in the rice seed germination test, but significant effects were observed with the rice shoot growth test (Table 2). Mesocosm 4 (land farm) soil never showed evidence of toxic effects to organisms survival, but soil collected in 1993 did possess some effects on rice shoot growth.

### Plant Stress Proteins

Physiologically, marked changes in patterns of stress proteins were observed in roots of rice seedlings treated with Mesocosm 3 (1992) soil extract, with near complete suppression at 100% soil extract. In particular, proteins at about 74, 70 and 56 KDa were suppressed in roots exposed to Mesocosm 3 soil extract, while they were present in roots exposed to reference Grid 1 soil extract and the blank (deionized water control). We also observed that several proteins were induced in roots treated with contaminated soils. For example, polypeptides of 34 KDa were induced in roots exposed to contaminated soil extracts from Grid 2 (asphalt waste storage pit area), Mesocosm 2 and Mesocosm 3 (1993). Mesocosm 3 (1993) soil extract which had stimulatory effects on root growth, induced polypeptides of 34 and 19 KDa in the roots. In this study proteins were induced when both shoot and root growth were inhibited and induction often occurred independent of other measurable toxic effects.

Table 2. Toxicity test results from exposure to either soil extracts or soil samples collected from grids (G1-G6) and mesocosms (M1-M6) at the Oklahoma Oil Refining Co. Superfund Waste Site. No observed effect concentration (NOEC) expressed as percent inhibition relative to controls.

Toxicity Test	Year	G1	G2	G3	G4	G5	G6	M1	M2	M3	M4	M5	M6
<i>Ceriodaphnia</i>	1992	NE	NE	NE	NE	NE	NE	NE	NE	*	NE	NE	NE
7-day survival <sup>c</sup>	1993	NE	NE	NE	NE	NE	NE	NE	100	**	NE	NE	NE
<i>Ceriodaphnia</i>	1992	NE	NE	NE	NE	22	NE	NE	78	*	NE	NE	NE
7-day repro <sup>c</sup>	1993	NE	NE	NE	NE	42	47	NE	100	**	NE	29	17
Rice seed 10-day germination <sup>s</sup>	1992	NE	NE	NE	NE	20	14	NE	NE	44	NE	20	14
	1993	NE	NE	10	8	24	NE	NE	NE	46	NE	NE	NE
Rice shoot 10-day dry wt <sup>s</sup>	1992	NE	NE	22	25	NE	NE	NE	31	81	13	NE	NE
	1993	NE	NE	21	30	24	NE	NE	21	71	NE	NE	NE
Rice root 5-day length <sup>c</sup>	1992	NE	NE	NE	NE	NE	NE	NE	24	91	NE	NE	NE
	1993	NE	NE	NE	NE	14	NE	NE	NE	19	NE	NE	18
Rice Root 5-day dry wt <sup>c</sup>	1992	NE	NE	27	NE	26	NE	NE	44	54	NE	NE	NE
	1993	NE	NE	NE	NE	NE	NE	NE	27	25	NE	NE	NE
Microtox <sup>c</sup>	1992	19	NE	24	20	58	29	25	NE	ε	NE	37	29
5 min. test	1993	24	NE	NE	NE	23	23	23	NE	φ	NE	18	29

<sup>c</sup>aqueous soil extract  
<sup>s</sup>soil

NE = no biologically significant effect in highest dilutions tested

\* = *Ceriodaphnia* 0% survival in 0.01% in M3 soil extract (1992).

\*\* = *Ceriodaphnia* 0% survival in 20% M3 soil extract (1993).

ε = Microtox 9% light inhibition in 1% M3 soil extract (1992).

φ = Microtox 26% light inhibition in 1% M3 soil extract (1993).

Table 3. LC50 (confidence interval), EC50, and NOEC responses caused by soil contaminants in mesocosms 2 (M2) and mesocosm 3 (M3), Oklahoma Oil Refining Co. Superfund Waste Site.

	Year	LC50*		EC50*		NOEC*	
		M2	M3	M2	M3	M2	M3
<i>Ceriodaphnia</i>	1992	NE	ε	-	-	NE	0.005
7-day survival <sup>c</sup>	1993	ε	12.8 (7.5-17.7)	-	-	50	5
<i>Ceriodaphnia</i>	1992	-	-	-	-	-	0.001
7-day repro <sup>c</sup>	1993	-	-	-	-	12.5	0.005
Fathead minnow	1992	NE	0.06 (.05-.08)	-	-	NE	0.01
7-day survival <sup>c</sup>	1993	NE	> 20	-	-	NE	5
Rice seed 10 day germination <sup>s</sup>	1992	NE	-	-	-	-	-
	1993	NE	-	-	-	-	-
Rice shoot 10 day dry weight <sup>s</sup>	1992	-	-	-	-	-	-
	1993	-	-	-	φ	-	-
Rice 5 day root length <sup>c</sup>	1992	-	-	NE	14 (6.7-25.4)	-	-
	1993	-	-	NE	-	-	-
Rice 5 day dry weight <sup>c</sup>	1992	-	-	NE	-	-	-
	1993	-	-	NE	-	-	-
Microtox <sup>c</sup>	1992	-	-	NE	> 5	NE	0.5
5 min. test	1993	-	-	NE	> 5	NE	0.5

\*% soil elutriate or soil

<sup>c</sup>soil elutriate

<sup>s</sup>soil

NE = no biological effect

ε = all or none response for *Ceriodaphnia* survival, could not calculate.

φ = Rice shoot dry weight never increased at lowest dilution of M3 soil tested

- = test not performed.

In addition to roots, leaves also showed changes in protein patterns when seedlings are exposed to petrochemical-contaminated soil extracts. A 98 Kda polypeptide was suppressed in leaves of plants exposed to Mesocosm 3 (1992) soil extract (Fig. 2). In comparison, a 94 KDa polypeptide was highly induced in leaves of plants grown in contaminated soils from Grid 3 and Mesocosm 3 (Fig. 3) compared to reference samples. Changes in several other stress proteins (e.g. 29 and 21 KDa polypeptides) were noted during these trials, with both induction and suppression being observed in response to exposure to toxic soil extracts. Protein biomarkers appeared to offer additional capabilities for determining if adverse effects still exist in the ecosystem in cases where toxicity tests or field tests may fail to detect ecologically significant effects.

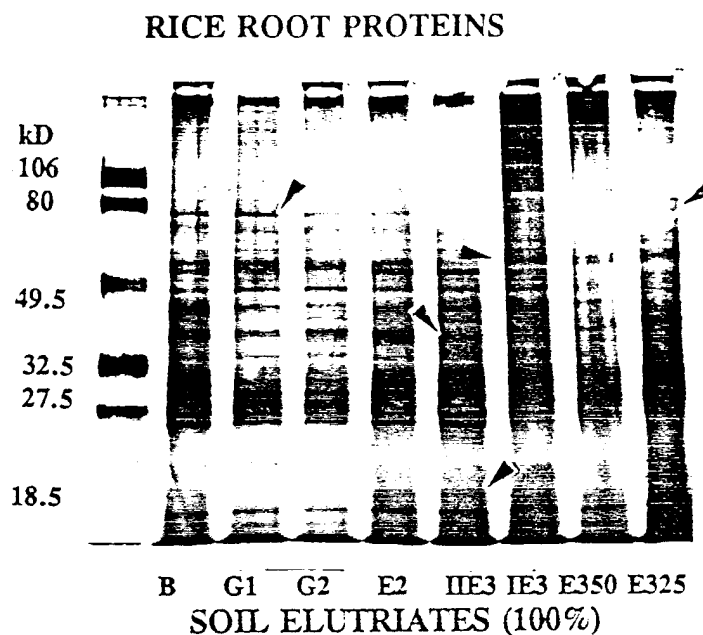


Fig. 2. Root proteins of rice plants after exposure to aqueous extracts of contaminated soil from grid (G1-G2) and mesocosm (E2-E325) sites.

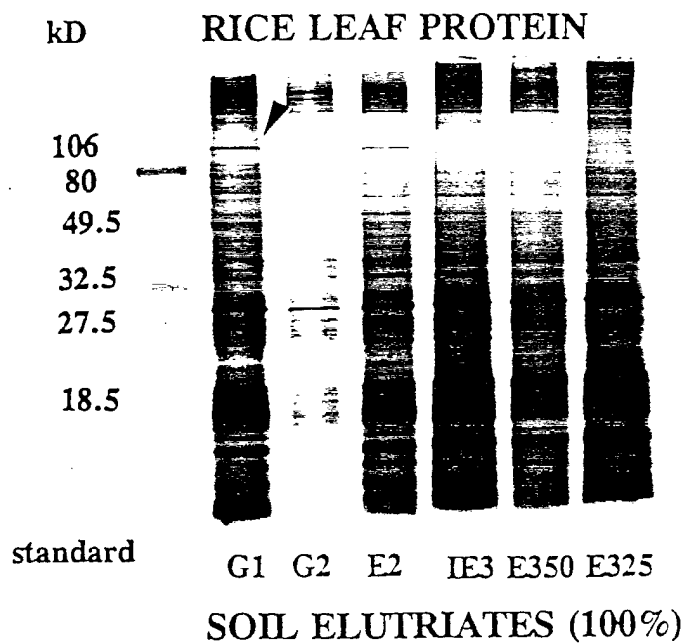


Fig. 3. Leaf proteins of rice plants after exposure to aqueous extracts of contaminated soil from grid (G1-G2) and mesocosm (E2-E325) sites.



### Microtox

Compared to *Ceriodaphnia* and fish bioassay tests of toxicity, the much utilized Microtox Assay System was found to be the least sensitive of all laboratory bioassays to the petrochemical contaminants that existed on the Cyril Oil Refinery Site (Tables 2 and 3). Though soil collected from Grids 3 and 4 (1992) caused significant light diminution when compared to Microtox diluent controls, the soil collected in 1993 caused no significant effect.

### Overview

The laboratory bioassay results indicated that selected sites at the refinery have contaminants that can pose threats to both aquatic and terrestrial communities. Mesocosm 3 was without question the most toxic of our study sites using these tests. *Ceriodaphnia* was the most sensitive species of all the bioassay organisms tested in this study. The bioassays also revealed, as did other in situ measures of toxicity, that toxicity was variable across seasons and years. For example, Mesocosm 2 soil collected in 1993 contained visibly greater quantities of gasoline and were more toxic to *Ceriodaphnia* than the soil collected in 1992. In contrast the 1993 Mesocosm 3 soil was less toxic to aquatic organisms than the previous year. We suspect that these temporal variations are due to climatic variability. In 1993 soil sampling in January was followed by heavy rains in the area which could have shifted the contaminants. The contaminants in the soil samples also possibly differed due to random sampling method.

In this study three reference sites were used to compare three contaminated sites. Sometimes the reference (uncontaminated) sites exhibited physical and chemical parameters that caused adverse effects to the test battery. As a result, we suggest that a laboratory control can be used for preliminary screening of the reference sites. In this study rice had shown sensitivity to the refinery site soil even though other bioassays did not, indicating that a phytotoxicity assay of some sort should be included in a test battery employed for these types of hazardous waste sites.

Table 4. Comparison of EPA's Chronic Toxicity Reference Levels (Toxic Characteristics Leachate Procedure; TCLP-CTR; CFR 1990) with contaminant levels found on study areas located in the vicinity of the Olahoma Refining Co. Superfund Waste Site, Cyril OK.

Constituent	CTR levels (mg/l)	Regulatory level (mg/l)	Maximum level detected in TCLP extracts from Cyril (mg/l)
Arsenic	0.05	5.0	2.8 (Mesocosm3, API separator ponds)
Cadmium	0.01	1.0	0.009 (Grid 4, waste oil land farm)
Chromium	0.05	5.0	1.0 (Mesocosm 3)
Lead	0.05	5.0	3.98 (Grid 4)

No chemical constituents analyzed in the TCLP extracts or the aqueous extracts of soils from the Cyril Refinery exceeded EPA regulatory guidelines (Table 4). However, toxicity tests of soil and soil leachates detected levels of contamination that ranged from highly lethal in acute short term tests to subtle but still significantly deleterious effects upon long term stability of the populations of feral rodents living on the site. EPA has made significant advances in

establishing regulatory limits to protect human health from significant levels of chemical constituents in contaminated soils that could leach into drinking water. However, as our study has shown, if ecological effects are given equal relevance with respect to regulating deleterious levels of contamination, then the 1990 TCLP guidelines are not adequately protecting the terrestrial and aquatic ecosystems adjacent to the contaminated waste sites.

## Monitoring of Wild Populations

### Mammalian Population Dynamics

Field monitoring of resident small mammal populations and community dynamics at Cyril has provided evidence of petrochemical-induced toxicity at the population level; suggesting that demographic indices are useful measures of ecotoxicity in terrestrial environments. Small mammal populations on the 6 study sites have been monitored since January 1991 (through August 1992) at 8 week intervals except in summer and winter where a 3 week interval was followed for 4 consecutive trapping periods (total 17 trapping bouts). Total biomass, sex ratios, reproduction, recruitment, survival, and density were measured as indices of population integrity. Community profiles included measurements of diversity, richness, and similarity.

During the course of the study, a total of 11 species of small mammals was captured, representing 9 rodents and 2 insectivores. House mice (*Mus musculus*), white-footed mice (*Peromyscus leucopus*), deer mice (*P. maniculatus*), and fulvous harvest mice (*Reithrodontomys fulvescens*) were seasonally abundant on most areas sampled. However, cotton rats (*Sigmodon hispidus*) represented the most abundant small mammal collected on all areas during the entire course of the study. Cotton rat density was seasonally variable and was significantly different between toxic and reference sites (Fig. 4). Greatest differences in density between toxic and reference sites was observed in 1991, especially in July and August when reference study sites supported greater densities than suspected toxic study sites. Differences were less obvious in 1992 although August results showed similar trends as in 1991.

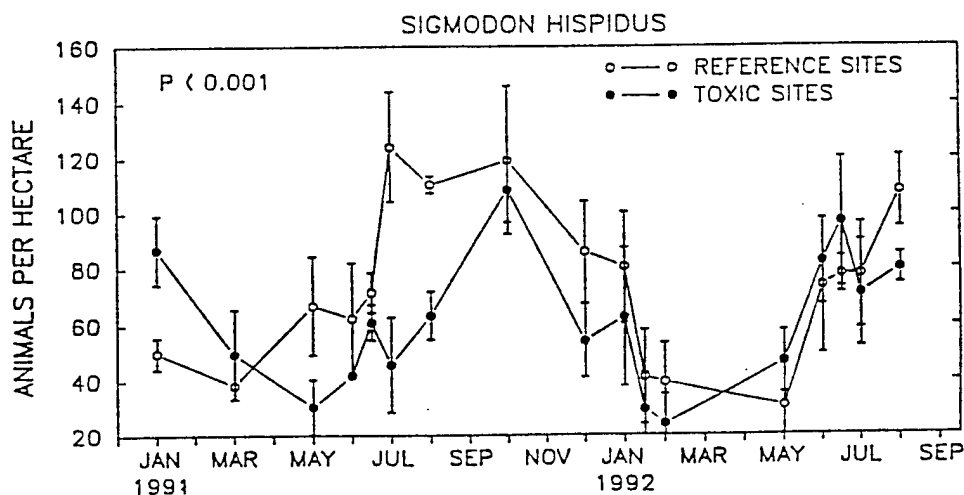


Fig. 4. Differences in mean ( $\pm$ SE) mark-recapture density estimates of cotton rat populations on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

Reproductive status of adult female cotton rats do not appear to indicate any differences between toxic and reference sites (Fig. 5). However, recruitment (as indicated by percentage of juvenile cotton rats in the trapable population, Fig. 5) trends closely resembled responses seen for density in this species. The lack of obvious differences in female reproductive activity coupled with lower recruitment patterns on toxic sites indicated possible differences in juvenile survival. Analysis of survival of all trapable cotton rats collected in the study do not appear to indicate any differences between toxic and reference sites (Fig. 6). However, these results represent a very cursory examination of survival. It is likely that differences in survival do exist within discrete age-classes. For example, differential survival of juvenile (both in utero and neonatal mortality) or aged animals may be possible among study sites. Further analysis of the existing data set will aid in determine if differential survival existed among specific age classes within populations.

Diversity of small mammal communities on toxic and reference sites showed a relatively high degree of divergence from May 1991 through February 1992 (Fig. 7). During this time, diversity was consistently higher on toxic sites compared to references due to the presence of a high number of house mice on toxic sites. It is difficult at this time to determine the significance of this difference. Increased numbers of house mice could represent a remnant population previously associated with the refinery. Another more interesting possibility is that house mice could be more resistant to contaminant exposure than their native counterparts and thus outcompeting them for resources. Similarity indices were included as a measure of the composition and relative abundance of species within communities (Fig. 8). Similarity indices indicated differences in the structure of small mammal communities existed between toxic and reference sites in summer samples; differentiation between sites was less obvious for winter samples.

Both population and community indices demonstrated sensitivity as ecosystem endpoint markers of contaminant exposure. The primary "lesion" at the population level appeared to be differential survival rates between toxic and reference sites, although at this time we are uncertain exactly which component of the population was most susceptible. Differences in density of cotton rat populations coincided nicely with the majority of immunological lesions observed in the same populations. Altered immune function resulting in increased susceptibility to infection and disease could be responsible for increased mortality on toxic sites.

Four collections of resident cotton rats were conducted (January and September 1991, March and September 1992), resulting in a total collection of 217 cotton rats from 6 areas (3 toxic and 3 reference sites) for physiological response profiling. Animals were returned to the laboratory where pathological, immunological, metabolic, and cytogenetic endpoints were measured. The results of these findings are described below:

### Pathologic Examinations

An important component of our in situ study was to explore the use of pathological examinations (gross and histologic) for measuring toxic exposure in resident small mammals. Gross pathologic examinations proved to be an integral part of our multiparameter model, where cotton rats from wild-caught populations inhabiting petrochemical-contaminated areas were observed to possess prominent morphological alterations in dental morphology. The normal color of their incisors is deep yellow-orange, which is imparted by a pigment produced by ameloblasts; abnormal incisors were pale and mottled. Sixty-one of 107 animals from contaminated sites and zero of 102 from reference sites had grossly abnormal teeth (Fig. 9); overall dental lesion incidence was 0% in cotton rats from all reference grids and 24%, 42% and 97% in rats from contaminated grids 2, 3, and 4 respectively. Microscopic examination of the undecalcified normal and abnormal incisors revealed that the enamel of the abnormal teeth was irregular, and lacked pigment, which was attributed to the degenerative and necrotic changes in the ameloblasts of abnormal incisors. We have correlated enamel lesions with fluoride toxicity.

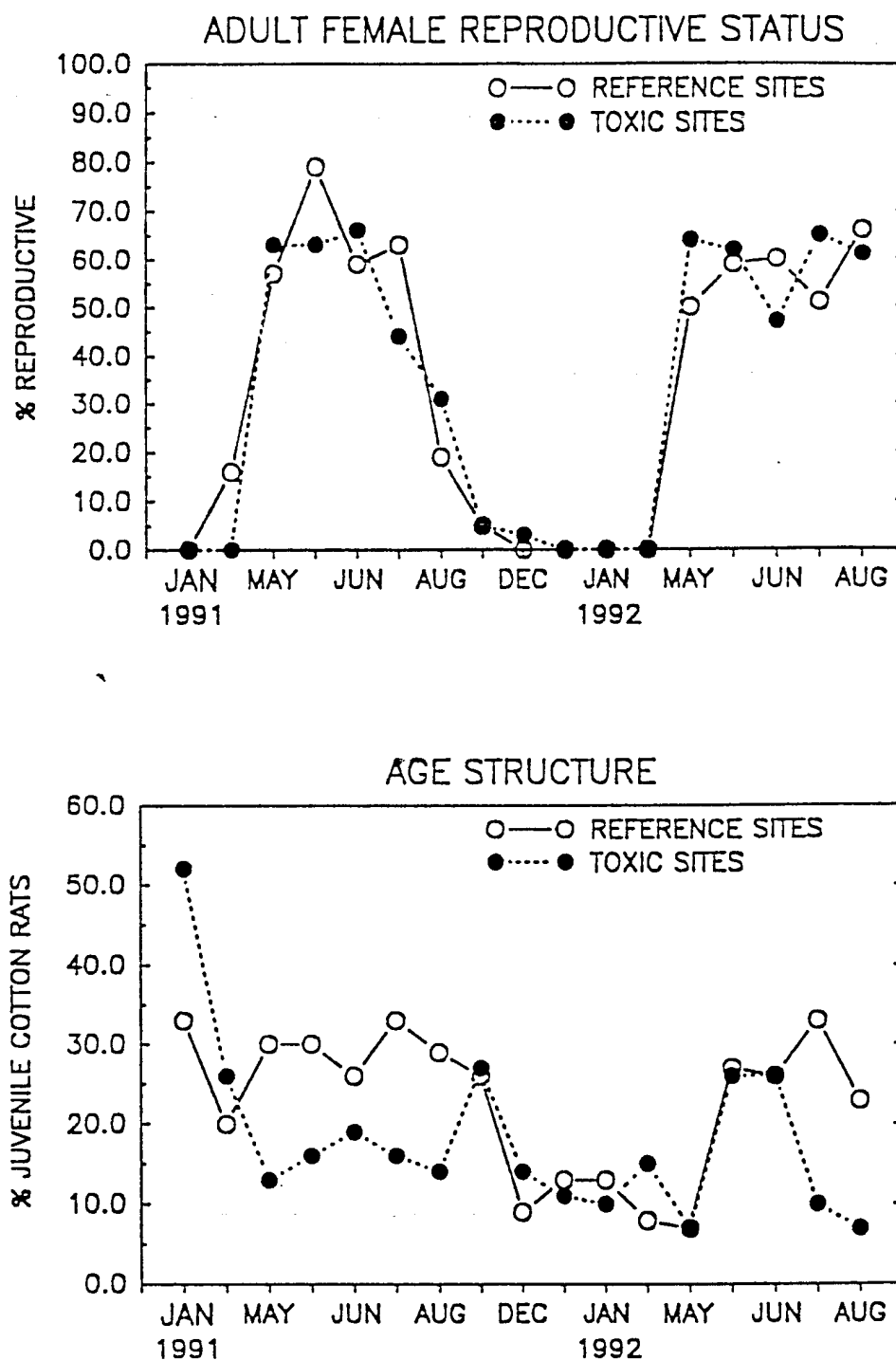


Fig. 5. Differences in mean percent reproductively active (pregnant or lactating) adult female cotton rats and percent juvenile ( $\leq 60$ g) cotton rats captured on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

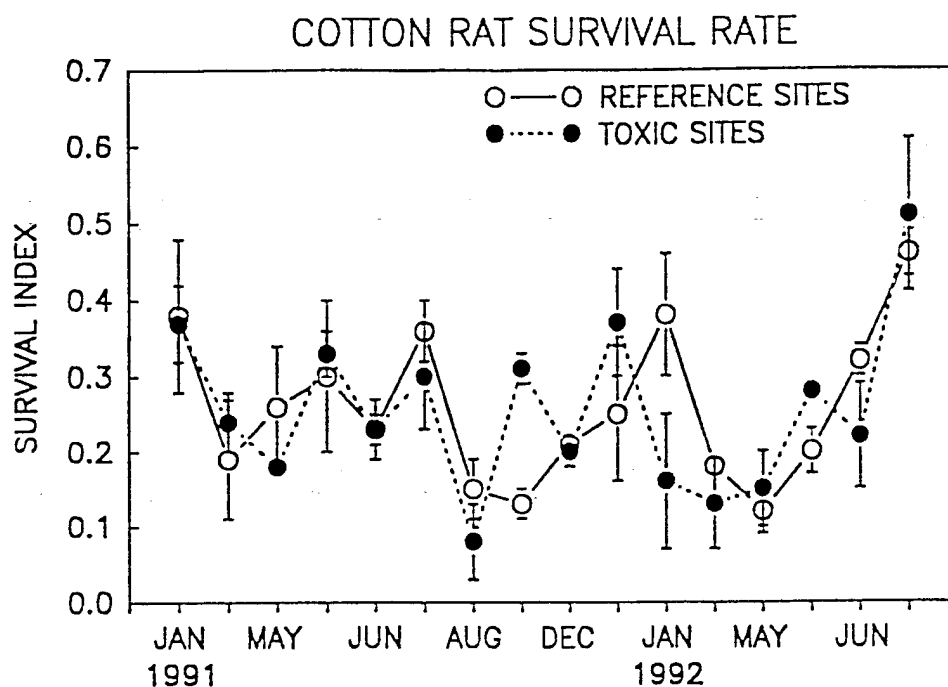


Fig. 6. Differences in mean finite survival rates of all trapable cotton rats on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to July 1992.

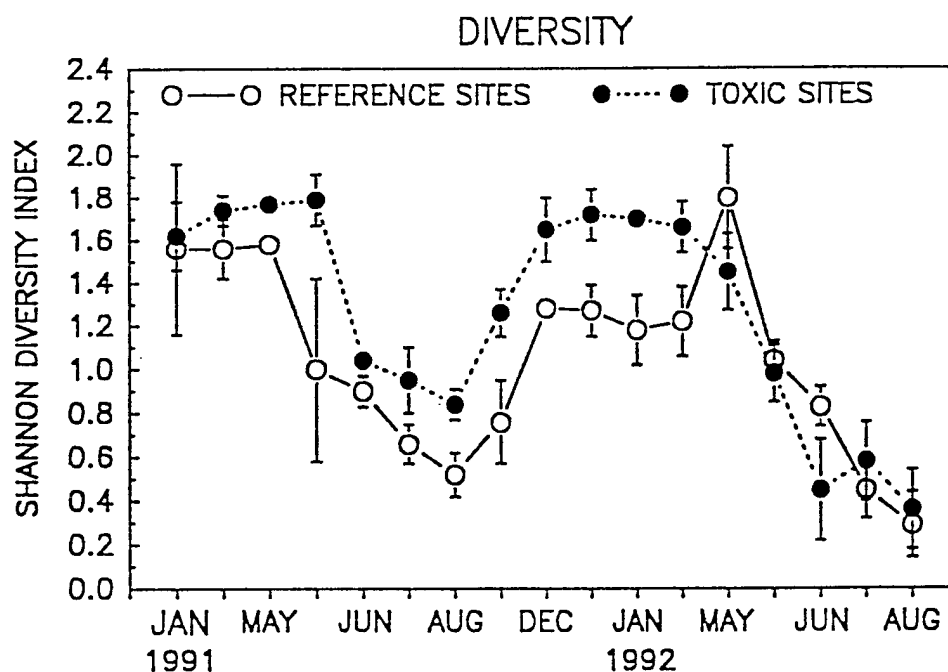


Fig. 7. Differences in mean diversity indices of small mammal communities on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma from January 1991 to August 1992.

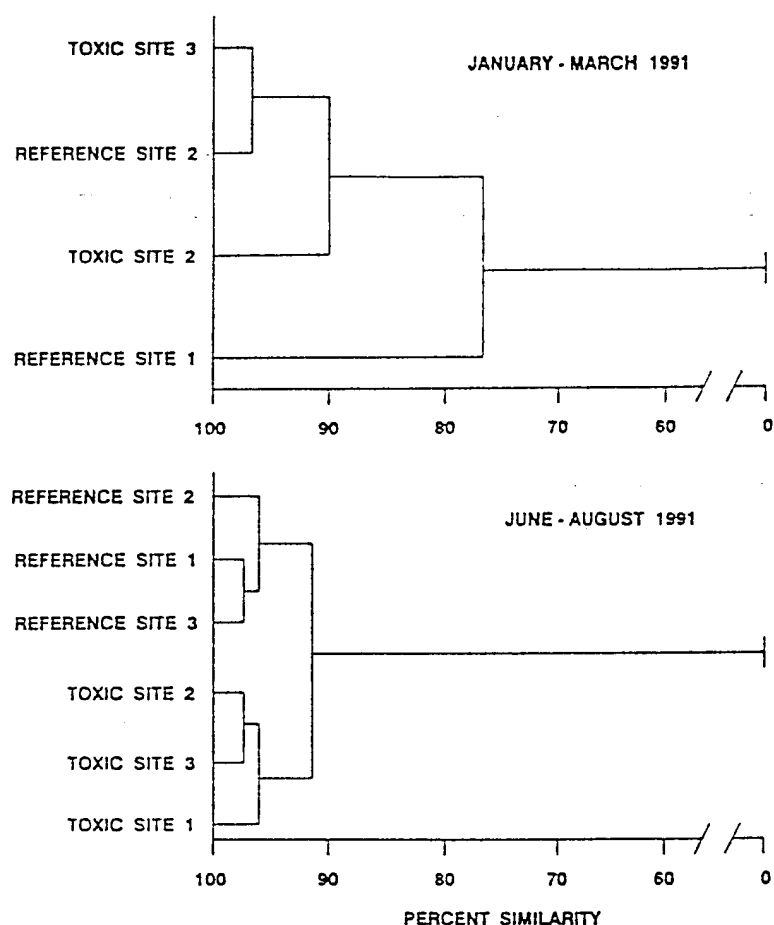


Fig. 8. Similarity in species composition of small mammal communities on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during winter 1991 (January and March pooled) and summer 1991 (June, July, and August pooled). Similarity is based on the species present and their relative proportion to each other within each community.

Bone fluoride concentrations measured, as a result of our morphologic findings ( $n=22$ ), was  $1657 \pm 527$   $\mu\text{g/g}$  (mean  $\pm$  s.e.) bone ash from rats showing severe lesions from contaminated sites compared to  $192 \pm 23$   $\mu\text{g/g}$  on reference sites.

In addition to evaluation of teeth tissues, histopathologic examinations of the soft tissues of cotton rats were conducted as well. The following tissues were examined histologically: brain, heart, lung, liver, kidneys, pancreas, testicle, adrenal gland, lymph node, small intestine, large intestine, urinary bladder, spleen, lymph node, and stomach. No lesions attributable to environmental contaminants on toxic sites were observed. A variety of microscopic lesions were observed in cotton rats from both reference and toxic study sites. Examples include non-suppurative interstitial nephritis, calcification of the urinary bladder (Chandra et al. 1993), and in aged laboratory-reared cotton rats we observed altered hepatocellular foci, a preneoplastic lesion (Paranjpe et al. 1993).

Our initial results provide strong evidence in support of using both gross and histologic examinations in assessing toxicity in situ with small mammalian residents. These results are only preliminary and further study will be needed to fully validate their usefulness.

### Immunotoxicity Evaluations

Unlike other studies which used controlled dosing protocols to examine the immunotoxicity of known compounds in wild rodents maintained in the laboratory, our results demonstrated the usefulness and response of immunological endpoints to contaminant exposure in a wild population of rodents simultaneously subjected to a plethora of environmental stressors routinely encountered under natural conditions.

As expected, a considerable amount of variation in immune responses were observed among and within populations over time. Several immune parameters, including complement levels, phagocytic activity of macrophages, and proliferative response of lymphocytes to the B and T cell mitogens, PWM and IL-2, have not been sensitive indicators of contaminant exposure (Table 5). Additionally, no single immune response endpoint showed significant differences between toxic and reference sites during every seasonal collection period. However, several trends were apparent from our results in this study. The majority of the differences were observed in the September collections compared to winter, possibly indicating a seasonal dependence in response linked to other extrinsic or intrinsic stressors. Also, all indices of leukocyte cellularity were typically lower on toxic compared to reference sites. T lymphocytes, as indicated by mitogenic response to Con A and Con A-based subtyping, appeared to be the most sensitive immune lesion. B lymphocytes, as indicated by mitogenic response to PWM and IgG-based subtyping, appeared to be relatively insensitive to contaminant exposure.

Immune organ indices of general condition showed significant variation between toxic and reference sites and among collection grids during September 1991 and 1992 collection periods (Table 6). Relative spleen mass of cotton rats was heavier and paired adrenals and popliteal lymph nodes were lighter in cotton rats collected on toxic sites in September 1991 and September 1992. Overall, kidney mass in September 1991 and kidney mass and liver mass in September 1992 were not significantly ( $P > 0.100$ ) different between toxic and reference sites although significant variation among the six collection grids was evident (Table 6).

A number of different leukocyte and erythrocyte indices showed significant variation between toxic and reference sites and among toxic and reference collection grids, typically reflecting the highest degree of sensitivity in September collection periods (Table 7). Total leukocyte, lymphocyte, and neutrophil counts were significantly lower in cotton rats collected from toxic sites compared to reference sites in January 1991. Total number of splenocytes was higher in cotton rats collected from toxic sites compared to references in September 1992, whereas cellularity of paired popliteal lymph nodes (total cells and cells per mg of node) was lower on toxic sites during the same period. Lymphocyte subtyping showed Con A positive T-lymphocytes to be significantly depressed in cotton rats collected from toxic sites compared to reference sites. Erythrocyte indices showed significant variation in September 1991 and September 1992 collections (Table 7). Erythrocyte counts and packed cell volume were significantly higher in September 1991 and erythrocytes lower in September 1992 in cotton rats collected from toxic sites compared to references. Depressed erythrocyte levels on toxic sites in September 1992 were accompanied by a significant increase in corpuscular volume and hemoglobin. Platelet counts were significantly ( $P < 0.040$ ) higher in cotton rats collected from toxic sites compared to reference sites in September 1992.

In addition to the above indices, a number of different functional measures of immunity were also assessed. In general, proliferative capacity of splenocytes, both innate (unstimulated) and Con A-stimulated, was the most consistent discriminator of immune dysfunction between cotton rats from toxic and reference sites (Figs. 10 and 11). In vitro proliferation of unstimulated splenocytes was significantly greater on toxic sites in September 1991 and September 1992 compared to reference sites (Fig. 10). T lymphocyte proliferation following stimulation with Con A at 5ug/ml culture in January 1991 and September 1992 and 40ug/ml culture in September 1991 was significantly higher for cotton rats collected from toxic sites compared to reference sites (Fig. 11).

Table 5. Mean (SE) values for immunological and condition variables of cotton rats collected from the Oklahoma Oil Refining Co. Superfund Waste Site, Cyril, OK that were not significantly different between reference and toxic study sites.

	n	Toxic grids	n	Reference grids
<b>Cellularity</b>				
Eosinophils (per ul)	48	905(174)	48	781(115)
Basophils (per ul)	48	25(9)	48	9(4)
Monocytes (per ul)	48	356(43)	48	324(56)
Lymphocytes (X)	48	51.0(2.3)	48	48.8(2.3)
Neutrophils (X)	48	36.4(1.1)	48	39.8(1.9)
Eosinophils (X)	48	8.3(0.1)	48	7.9(0.9)
Basophils (X)	48	0.3(0.4)	48	0.1(0.04)
Monocytes (X)	48	4.1(0.1)	48	3.4(0.5)
Splenocytes/mg spleen $\times 10^3$	98	349(18)	99	373(16)
IgG positive splenocytes (X)	47	58.7(1.0)	49	57.8(1.0)
Hemoglobin (g/dl)	60	13.7(0.2)	60	13.6(0.2)
Mean corpuscular hemoglobin concentration (X)	60	31.7(0.2)	60	31.7(0.2)
<b>Immunological Immunity</b>				
Complement activity (CH50)	108	3955(259)	108	3819(243)
<b>Non-specific Immunity</b>				
Number of latex beads engulfed/ phagocytically active macrophage	30	1.96(0.12)	28	1.96(0.12)
Percentage of total macrophages phagocytizing latex beads	30	21.0(2.6)	28	21.2(2.9)
Percent natural killer cell tumoricidal activity	60	51.9(2.7)	60	52.9(2.6)
Mitogen-induced splenocyte proliferation (ABS 570/630 nm)				
Pokeweed mitogen (0.625 ug/ml culture)	101	0.141(0.016)	102	0.138(0.015)
Pokeweed mitogen (1.25 ug/ml culture)	100	0.128(0.017)	102	0.119(0.014)
Interleukin-2 (40 U/ml culture)	83	0.145(0.015)	83	0.172(0.017)
<b>In vivo cellular immunity</b>				
24-hr hypersensitivity (X)	90	122(10)	90	129(9)



Table 6. Mean (SE) relative mass (S) of selected organs of cotton rats collected from replicated toxic and reference grids at the Oklahoma Oil Refining Co. Superfund Waste Site, Cyril, OK.

Collection date	Toxic grids				Reference grids			Overall means	
	Grid 2	Grid 3	Grid 4	Grid 1	Grid 5	Grid 6	Toxic	Reference	
<u>September 1991</u>									
Spleen <sup>a</sup>	2.65(0.20) <sup>ab</sup>	2.94(0.29) <sup>a</sup>	2.29(0.15) <sup>b</sup>	1.65(0.16) <sup>c</sup>	2.21(0.13) <sup>b</sup>	2.24(0.19) <sup>b</sup>	2.62(0.13) <sup>*</sup>	2.05(0.10)	
Adrenals	0.27(0.01) <sup>a</sup>	0.29(0.02) <sup>ac</sup>	0.31(0.03) <sup>abc</sup>	0.31(0.02) <sup>abc</sup>	0.34(0.02) <sup>bc</sup>	0.36(0.03) <sup>b</sup>	0.29(0.01) <sup>*</sup>	0.34(0.01)	
Kidneys	7.20(0.20) <sup>a</sup>	7.20(0.20) <sup>a</sup>	6.50(0.20) <sup>c</sup>	7.40(0.10) <sup>ab</sup>	7.80(0.20) <sup>b</sup>	7.40(0.20) <sup>ab</sup>	7.00(0.10) <sup>**</sup>	7.50(0.10)	
<u>September 1992</u>									
Spleen	2.50(0.33) <sup>a</sup>	2.44(0.15) <sup>a</sup>	2.32(0.19) <sup>a</sup>	2.32(0.23) <sup>a</sup>	1.98(0.16) <sup>a</sup>	1.90(0.34) <sup>a</sup>	2.42(0.13) <sup>*</sup>	2.07(0.15)	
Popliteal nodes	0.18(0.03) <sup>a</sup>	0.14(0.03) <sup>a</sup>	0.13(0.02) <sup>a</sup>	0.17(0.03) <sup>a</sup>	0.22(0.05) <sup>a</sup>	0.20(0.03) <sup>a</sup>	0.15(0.01) <sup>*</sup>	0.20(0.02)	
Kidneys	7.70(0.20) <sup>a</sup>	7.30(0.30) <sup>ab</sup>	6.80(0.30) <sup>b</sup>	7.50(0.20) <sup>ab</sup>	7.80(0.20) <sup>a</sup>	7.70(0.20) <sup>a</sup>	7.30(0.20) <sup>**</sup>	7.70(0.10)	
Liver	37.7(0.9) <sup>a</sup>	33.4(1.1) <sup>b</sup>	32.3(1.1) <sup>b</sup>	34.1(1.3) <sup>b</sup>	33.2(0.9) <sup>b</sup>	35.3(1.3) <sup>ab</sup>	34.5(0.7) <sup>**</sup>	34.2(0.7)	

S mg/g of body mass

<sup>a</sup> Means among grids with the same letter are not significantly different at  $P \leq 0.05$

\* Overall mean of toxic grids significantly different from reference grids at  $P \leq 0.09$

\*\* Not different from reference grids at  $P \geq 0.10$

Table 7. Mean (SE) cellularity values for blood and selected immune organs of adult cotton rats collected from replicated toxic and reference grids at the Oklahoma Oil Refining Co. Superfund Waste Site, Cyril, OK.

Collection date	Toxic grids			Reference grids			Overall means	
	Grid 2	Grid 3	Grid 4	Grid 1	Grid 5	Grid 6	Toxic	Reference
JANUARY 1991								
Leukocytes ( $\times 10^3/\mu\text{l}$ ) <sup>§</sup>	ND	4.43(0.31) <sup>a</sup>	4.84(0.50) <sup>a</sup>	6.68(0.48) <sup>b</sup>	7.98(1.10) <sup>b</sup>	ND	4.66(0.31) <sup>a</sup>	7.30(0.58)
Lymphocytes ( $\times 10^3/\mu\text{l}$ )	ND	2.43(0.37) <sup>a</sup>	2.87(0.43) <sup>ac</sup>	3.63(0.39) <sup>bc</sup>	4.35(0.61) <sup>b</sup>	ND	2.68(0.29) <sup>a</sup>	3.97(0.36)
Neutrophils ( $\times 10^3/\mu\text{l}$ )	ND	1.57(0.30) <sup>ac</sup>	1.47(0.23) <sup>a</sup>	2.60(0.19) <sup>b</sup>	2.93(0.67) <sup>bc</sup>	ND	1.51(0.18) <sup>a</sup>	2.76(0.32)
SEPTEMBER 1991								
Erythrocytes ( $\times 10^6/\mu\text{l}$ )	5.63(0.30) <sup>a</sup>	5.25(0.25) <sup>a</sup>	5.42(0.32) <sup>a</sup>	4.67(0.22) <sup>a</sup>	4.91(0.32) <sup>a</sup>	4.88(0.24) <sup>a</sup>	5.43(0.16) <sup>a</sup>	4.82(0.15)
Packed cell volume (%)	42.8(1.5) <sup>abc</sup>	44.6(0.7) <sup>bc</sup>	45.3(1.0) <sup>b</sup>	41.1(1.7) <sup>ac</sup>	39.3(1.0) <sup>a</sup>	41.6(1.4) <sup>ac</sup>	44.2(0.7) <sup>a</sup>	40.6(0.8)
Fluorescein-Concanavalin A positive splenocytes (%)	71.8(2.3) <sup>a</sup>	74.4(1.9) <sup>a</sup>	75.0(2.1) <sup>a</sup>	77.3(2.6) <sup>a</sup>	76.1(2.8) <sup>a</sup>	77.7(2.1) <sup>a</sup>	73.7(1.2) <sup>a</sup>	77.0(1.4)
SEPTEMBER 1992								
Erythrocytes ( $\times 10^6/\mu\text{l}$ )	6.61(0.28) <sup>a</sup>	6.35(0.25) <sup>a</sup>	6.55(0.18) <sup>a</sup>	6.61(0.22) <sup>a</sup>	7.04(0.16) <sup>a</sup>	6.97(0.21) <sup>a</sup>	6.50(0.13) <sup>aa</sup>	6.87(0.12)
Mean corpuscular volume ( $\mu^3$ )	66.3(0.8) <sup>ab</sup>	67.0(1.0) <sup>a</sup>	67.4(1.3) <sup>a</sup>	66.2(0.8) <sup>ab</sup>	64.1(1.1) <sup>b</sup>	63.8(0.9) <sup>b</sup>	66.9(0.6) <sup>aa</sup>	64.7(0.6)
Mean corpuscular hemoglobin (pg)	21.2(0.3) <sup>ab</sup>	21.6(0.4) <sup>a</sup>	21.4(0.5) <sup>a</sup>	21.0(0.3) <sup>ab</sup>	20.3(0.4) <sup>b</sup>	20.2(0.3) <sup>b</sup>	21.4(0.2) <sup>a</sup>	20.5(0.2)
Platelets ( $\times 10^3$ )	618(77) <sup>a</sup>	676(73) <sup>a</sup>	607(66) <sup>a</sup>	549(59) <sup>a</sup>	569(49) <sup>a</sup>	485(30) <sup>a</sup>	633(41) <sup>a</sup>	534(27)
Spleen cellularity ( $\times 10^6$ )	80.5(14.8) <sup>a</sup>	76.8(13.5) <sup>a</sup>	95.8(12.0) <sup>a</sup>	70.2(7.4) <sup>a</sup>	70.6(10.0) <sup>a</sup>	57.9(12.3) <sup>a</sup>	84.4(7.7) <sup>aa</sup>	66.2(5.7)
Paired popliteal node cellularity ( $\times 10^6$ )	21.3(4.4) <sup>a</sup>	16.9(4.5) <sup>a</sup>	19.8(4.9) <sup>a</sup>	25.4(6.2) <sup>a</sup>	26.6(6.6) <sup>a</sup>	26.7(6.6) <sup>a</sup>	19.4(2.6) <sup>a</sup>	26.2(3.6)
Paired popliteal node cellularity ( $\times 10^6/\text{mg node}$ )	1.04(0.10) <sup>ac</sup>	0.92(0.08) <sup>a</sup>	1.02(0.09) <sup>a</sup>	1.36(0.16) <sup>bc</sup>	1.22(0.12) <sup>abc</sup>	1.39(0.13) <sup>b</sup>	0.99(0.05) <sup>a</sup>	1.32(0.08)

n = 10 for all means except leukocytes, lymphocytes, neutrophils for grids 3 and 5 where n = 8 and 9, respectively; erythrocytes in September 1991 for grid 1 where n = 9; packed cell volume for grids 1-3 and grid 6 where n = 9 and 8, respectively

<sup>§</sup>Means among grids with the same letter are not significantly different at  $P \leq 0.05$

<sup>a</sup>Overall mean of toxic sites significantly different from reference sites at  $P \leq 0.05$

<sup>aa</sup>Overall mean of toxic sites significantly different from reference sites at  $P \leq 0.08$

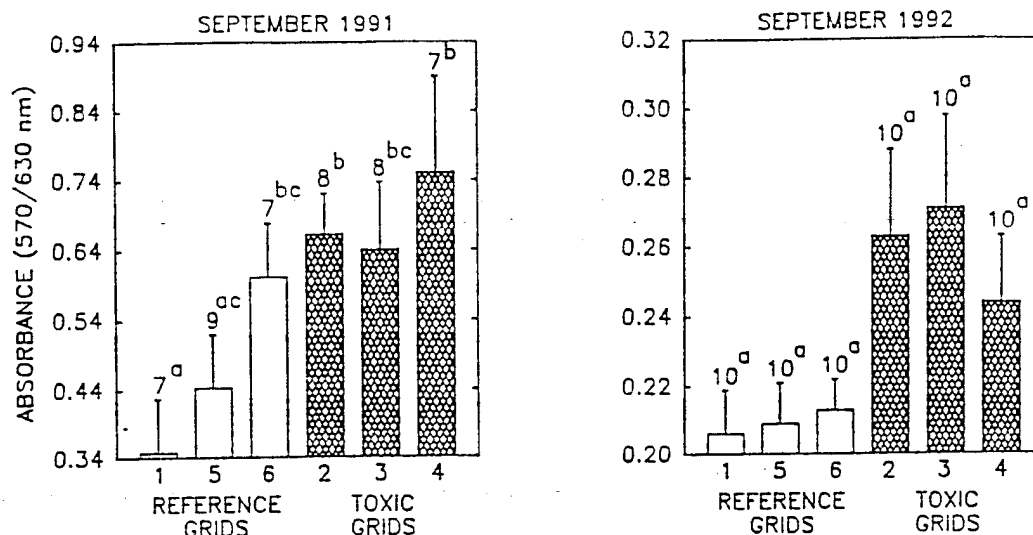


Fig. 10. Differences in unstimulated proliferative response (mean $\pm$ SE) of splenocytes (72-hr culture) from cotton rats collected on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during September 1991 and 1992. Values represent absorbance of solubilized formazan granules produced by splenocyte reduction of tetrazolium salt. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ( $P \leq 0.10$ ).

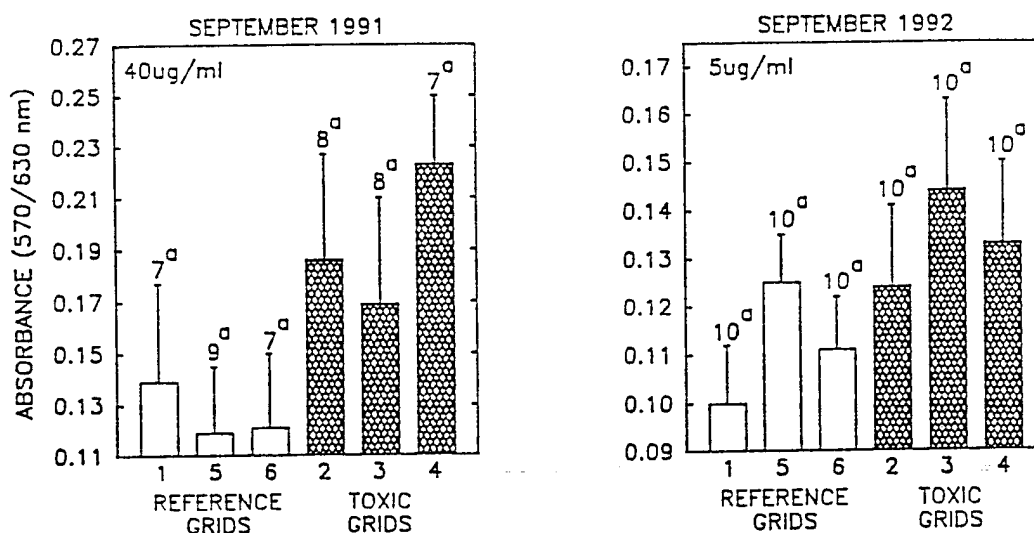


Fig. 11. Differences in mitogen-induced proliferative response (mean $\pm$ SE) of splenocytes (72 hr culture) from cotton rats collected on replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during January and September 1991 and September 1992. Cells were stimulated in vitro with either 5 or 40 ug/ml of culture Concanavalin A lectin. Values represent corrected absorbances (stimulated minus unstimulated cultures) of solubilized formazan granules produced by splenocyte reduction of tetrazolium salt. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ( $P \leq 0.10$ ).

In vivo cell mediated immune response, as measured by a 24-hr hypersensitivity response to an intradermal injection of phytohemagglutinin, was significantly higher in cotton rats collected from reference sites than toxic sites in March 1992 (Fig. 12). Metabolic activity of peritoneal macrophage populations showed a significant degree of variation among collection grids in January 1991 and September 1992 (Fig. 13). There appeared to be a definite seasonal trend in the degree of immunotoxicity. Late summer and autumn represents a stressful period for most small mammals due to reproduction demands, high densities, and nutritional stress. Possible interactions between xenobiotic stressors and other seasonally dependant natural stressors (e.g., reproduction) could have acted synergistically and enhanced an otherwise essentially benign toxic effect. Similar types of interactions have been shown by other investigators, where mice were subjected to several combinations of food, water, pathogens, and immunotoxins.

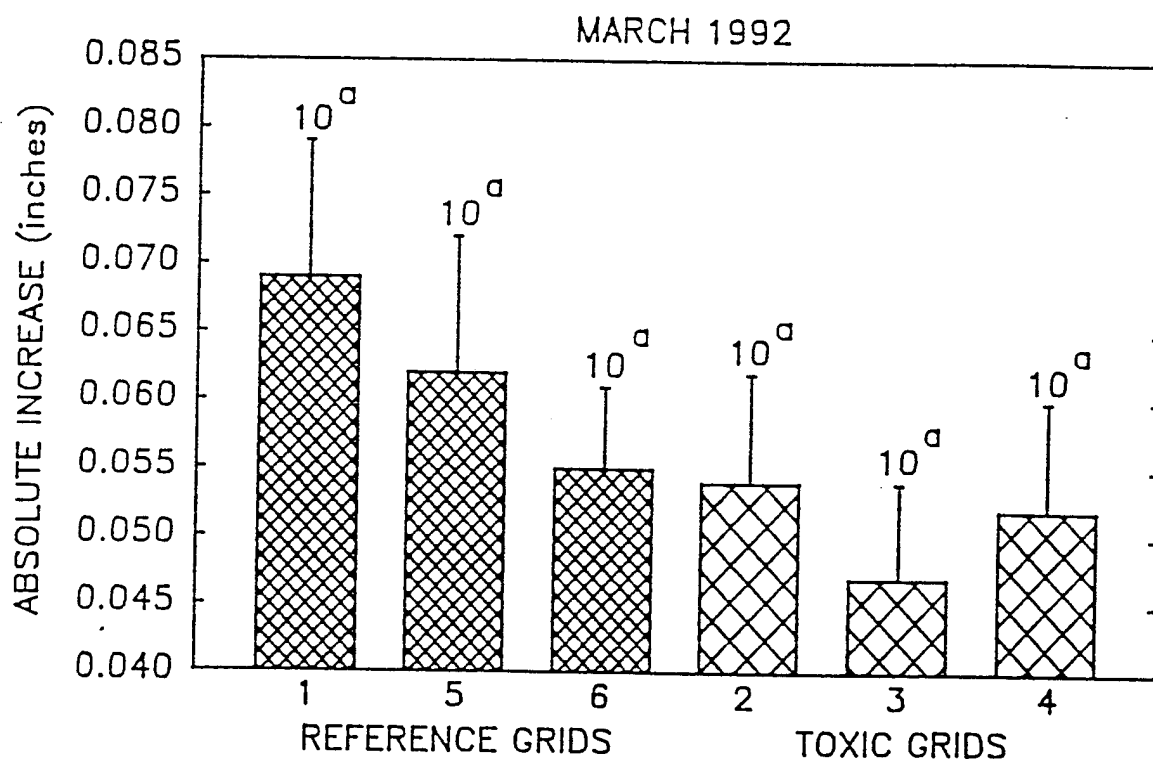


Fig. 12. Differences in mean ( $\pm$ SE) hypersensitivity response as reflected by the absolute increase (postimmune minus preimmune thickness) in skin fold thickness (inches) 24 hr after an intradermal injection of phytohemagglutinin (250 ug protein) in cotton rats. Animals were collected from replicate reference and toxic sites at an abandoned oil refinery in March 1992. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ( $P \leq 0.10$ ).

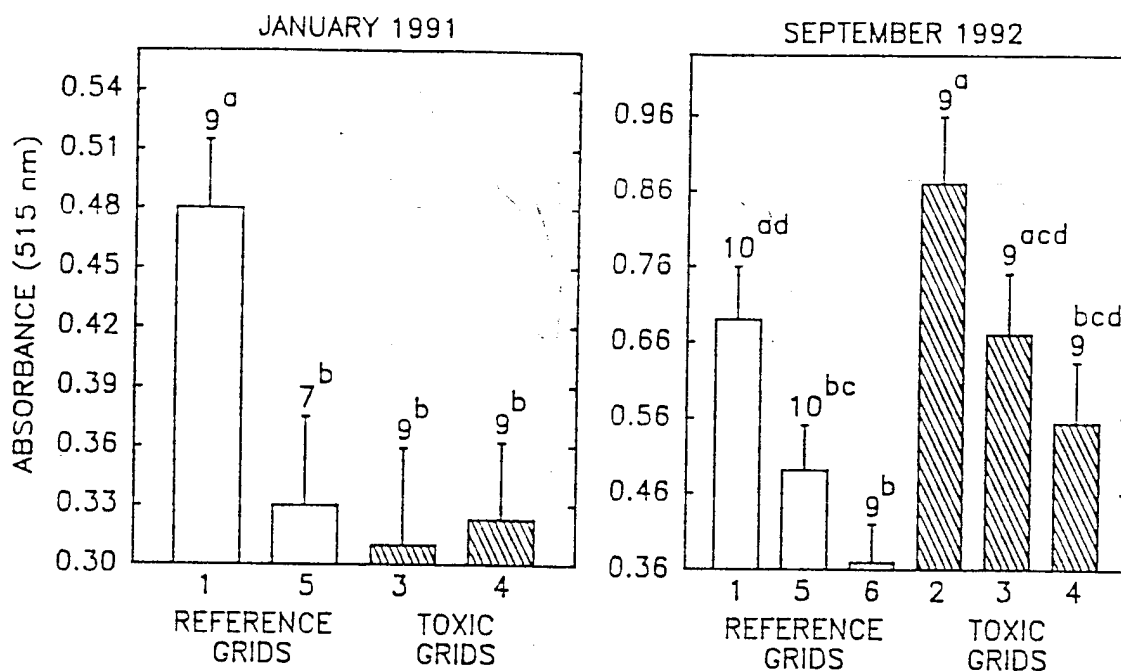


Fig. 13. Differences in mean ( $\pm$ SE) metabolic activity of peritoneal macrophages shown as absorbance values of cell cultures following reduction of nitroblue tetrazolium dye to formazan after 15 min incubation. Cells were recovered from cotton rats collected from replicate reference and toxic sites at an abandoned oil refinery in Oklahoma during January 1991 and September 1992. Values above bars represent sample size (n) and common letters denote no statistical difference between grids ( $P \leq 0.10$ ).

Increased *in vitro* splenocyte proliferation in unstimulated and stimulated (T cell mitogen, Con A) cultures were the most consistent indicators of immune dysfunction in cotton rats collected from toxic sites. It is unclear what mechanism is promoting the increase in blastogenesis (or metabolic activity), especially in the unstimulated cultures, although several possibilities exist. Regulatory activity of the immune system may be impaired with possible selective depletion and/or functional impairment of suppressor T cells. Lymphocytes may be stimulated to secrete factors which promote cell proliferation. Contaminants may alter cell surface markers, thereby rendering them antigenic in nature and eliciting an *in vivo* immune response against the transformed cells. At this point it is difficult to determine the exact mechanism(s) which would require more elaborate immunological assays. T suppressor cell generation is known to be sensitive to mercury exposure. Also, depletion of peripheral cells can lead to enhanced proliferation and differentiation of the pluripotent stem cell pool as a mechanism to replenish the depleted populations. Other studies have demonstrated the stimulatory effects of lead on lymphocyte blastogenesis, although most studies concur that lead suppresses proliferation. It is apparent that *in situ* monitoring can detect a variety of immune system lesions; the possible interactions between the numerous toxicants present on the sites and the impacts of, and interactions with, other extrinsic and intrinsic stressors which resident rodents are undoubtedly exposed to may be contributing factors.

#### Hepatic Cytochrome P450 Induction Studies

Analysis of methoxyresorufin (MROD) and ethoxyresorufin (EROD) O-dealkylase activity was performed on hepatic microsomes from wild caught cotton rats using methods adapted in our laboratory (Novak and Qualls 1989, Elangham et al. 1991, Burke et al. 1985). Evaluation of hepatic microsomal EROD and MROD was performed on *S. hispidus* collected from the Cyril Oil Refinery Site. We observed significant induction of both EROD and MROD activity in animals collected *in situ* from contaminated areas, especially during late summer

(September) collections (contaminated versus reference sites  $P < 0.01$ ). The degree of exposure was reflected in the magnitude of EROD and MROD induction, which was most prominent on contaminated grids 3 (API oil separator pond system) and 4 (oil process waste soil farming area) (Fig. 14). We hypothesize that observed seasonal differences in detoxication enzyme activity are associated with greater volatilization of hydrocarbons during warmer summer periods. These data once again support our contention that seasonal sampling is critical in reducing uncertainty and also increases accuracy of risk assessments.

As part of this project, we have developed and are currently standardizing automated assays for EROD and MROD using a Fluoroskan II automated multiwell plate reader with kinetic capabilities. It is estimated that this will increase productivity 20 fold, resulting in a marked saving in labor. In addition there will be an estimated decrease in reagent cost of more than 50% per assay.

### EROD Cyril, Oklahoma

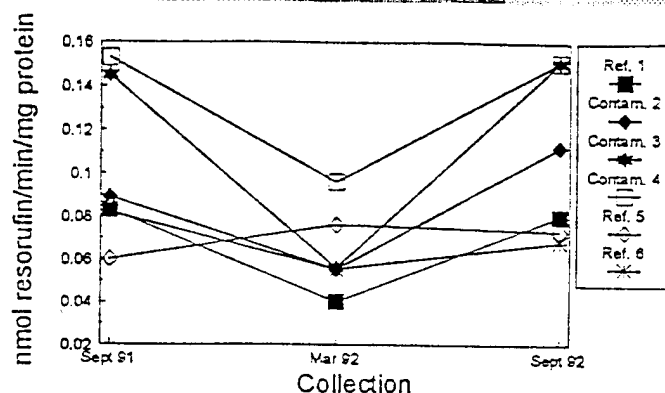


Fig. 14. Evaluations of ethoxyresorufin o-deethylation (EROD) by hepatic microsomes of cotton rats collected from the Oklahoma Refining Co. Superfund Waste Site. Note elevated EROD in September collections on contaminated sites 3 and 4. For combined data of reference vs. control  $P < 0.001$  for summer.

### Genotoxicity Evaluations

For each animal returned to the laboratory, chromosomes were extracted from bone marrow for analysis of mitotic metaphase chromosome aberrations, spleens were disaggregated and fixed for flow cytometric analysis of nuclear DNA content variation, and liver biopsies and thigh muscle biopsies were frozen in liquid nitrogen for analysis of DNA strand-breaks using agarose electrophoresis and scanning densitometry.

Chromosomal aberration analysis is the most fully developed assay for examination of genetic damage in wild mammal populations (McBee and Bickham, 1990). Fifty bone marrow metaphase chromosome preparations from each rat were examined for six classes of chromosomal aberrations: chromatid breaks, chromosome breaks, acentric fragments, rings, dicentric chromosomes, and translocation figures. Data were converted to number of aberrant cells per individual and number of lesions per cell for statistical analysis.

Animals collected during the first sampling period from the contaminated grids had fewer aberrant cells per individual and lesions per cell than did animals collected from the reference grids; however, the only statistically significant difference between pooled contaminated grid animals and reference site animals was in the number of acentric fragments.

For the fall 1991 trapping period, mean number of aberrant cells per individual was 2.33, 0.85, and 1.50 for the three contaminated grids and 2.55, 2.55, and 2.12 for the three reference grids. Mean number of lesions per cell was 0.06, 0.02, and 0.04 from the contaminated grids and 0.07, 0.05, and 0.050 for the three reference grids.

During the spring 1992 trapping period, more damage was observed in animals from both the contaminated grids and the reference grids; however animals from the contaminated grids had significantly more damage than animals from reference grids. Mean number of aberrant cells per individual was 3.5, 3.25, and 3.70 for the contaminated grids, and 2.40, 2.11, and 1.40 for the reference grids. Mean number of lesions per cell was 0.10, 0.09, and 0.11 for the contaminated grids and 0.05, 0.05, and 0.03 for the reference grids. Lesions per cell ( $p = 0.026$ ) and aberrant cells per individual ( $p = 0.046$ ) were both significantly greater in animals from contaminated grids than in animals from reference grids, largely due to a significantly greater ( $p = 0.002$ ) number of chromosomal breaks. In a grid-by-grid analysis, grid 3 (contaminated) had significant more translocation figures than grids 4 (contaminated), 5, and 6 (both reference). Grid 4 had significantly more acentric fragments and chromosome breaks than grids 5 and 6. Grids 2 and 4 (both contaminated) had significantly more chromatid breaks than grids 1 and 5 (both reference).

For the fall 1992 trapping period, animals from contaminated grids again showed significantly more chromosome damage than animals from reference grids. Mean number of aberrant cells per individual were 4.5, 3.2, and 3.3 for the contaminated grids and 1.8, 1.8, and 1.6 for the reference grids and mean number of aberrant cells per individual at contaminated grids was significantly greater ( $p = 0.007$ ) than in animals from the reference grids. Mean numbers of lesions per cell were 0.11, 0.08, and 0.07 for contaminated grids and 0.05, 0.04, and 0.04 for reference grids. Again, animals from the contaminated grids were significantly different ( $p = 0.011$ ) from animals from the reference grids. In a grid-by-grid analysis, grid 2 (contaminated) had significantly more chromosome breaks than all other grids; grid 4 (contaminated) had significantly more chromatid breaks than grids 1, 5, and 6 (all reference).

Flow cytometry has been used to demonstrate greater variation in nuclear DNA content and possible aneuploidy in spleen cells of wild mammals. Flow cytometric analysis included generation of over 60 standard DNA histograms and over 300 DNA histograms from wild cotton rats for each trapping period. Number-coded samples were analyzed using a Partec Pas-II fluorescent flow cytometer calibrated with a commercial standard preparation of calf thymocyte nuclei. For each rat, spleen cells were macerated and fixed in ethanol before staining with DAPI. Data from five replicates of 20,000 spleen cells each were collected and averaged for each animal. Variables derived from the averaged DNA histograms for each animal were analyzed statistically using Mann-Whitney U.

The mean coefficient of variation (CV) for nuclear DNA content of spleen cells from animals sampled during the fall 1991 trapping period ranged from a low (indicating little variability from cell to cell and therefore few genetic lesions) of 1.69 at grids 1 and 5 to a high of 1.83 at grid 2. Mean channel of the  $G_1$  peak, a relative measure of DNA content, was highest at grid 6 with a value of 64.83 which translated into approximately 9.5 pg/cell. Channel number was lowest at grid 2, with a value of 62.59 which translates into approximately 9.1 pg/cell. Grid 2 had a significantly higher CV compared to grids 1 and 5; grid 4 had a significantly higher CV compared to grid 5; no other CV comparisons were significantly different among grids. Grid 2 also had a significantly lower mean channel value than grids 5 and 6; grid 3 was significantly lower than 6; and grid 4 was significantly lower than grids 5 and 6.

The same general trends were apparent in animals collected during the spring 1992 trapping period. The three contaminated grids (2, 3, and 4) had higher CVs than the three reference grids (1, 5, and 6). The highest CV (2.66) occurred at grid 2 and the lowest CV (1.69) at grid 1. However, there were no statistically significant differences among grids for CV during the spring 1992 trapping period. Lower mean channel values were no longer apparent in animals from the contaminated sites for the spring 1992 trapping period either. Values ranged from a low of 59.95 at grid 5 which translated to approximately 8.9 pg/cell, to a high at grid 1 of

61.65 which translated to approximately 9.2 pg/cell. Grids 1 and 5 were significantly different from each other but significant differences were not found among the other grids.

During the fall 1992 trapping period, CVs were elevated for all grids compared to previous trapping periods. The sharp difference in higher CVs at contaminated grids is no longer apparent. No significant differences in CV were found among grids. The pattern of lower mean channel values at contaminated sites was seen again. The highest value was at grid 6 with a value of 63.48 which translated to approximately 9.3 pg/cell. The lowest value was at grid 2 with 61.16 or approximately 9.1 pg/cell. No significant differences for mean channel value were found among grids.

A proliferation index (PI--George, et al., 1991) is an indication of the amount of DNA synthesis in a population of cells calculated as a ratio of S plus G<sub>2</sub>M regions in a histogram divided by the mean value for these regions in all standards. Values greater than one indicate a proliferating cell population. Proliferation indices ranged from 0.83 at grid 2 in Fall 1992 to 1.14 at grid 1 in Fall 1991. The average PI for all contaminated grids was 0.94 and for all reference grids was 1.02; however, there were no statistically significant differences among grids over all three trapping periods.

## Mesocosm Experimental Trials

### General

One of the major objectives of this study was to determine the efficacy of applying our multiparameter model to animals housed within enclosures over specific sites of contamination. This was deemed an important component of the overall study because many areas contaminated with petrochemicals are not adequately vegetated to support natural rodent populations. Under these conditions, one may construct small enclosures directly over the sites of interest, thus permitting the investigator to introduce animals to the contaminated environment for a specified period of time. We successfully completed a series of eight-week in situ exposure trials, one in the winter of 1992 and one in the summer of 1992. Six mesocosms, 3 suspected contaminated and 3 reference locations, were utilized in both trials. The use of mesocosms to monitor or assess ecotoxicity has proved to be quite useful due to the greater experimental control that they afford. Animals of known age and exposure history can be placed upon a site of known contaminant composition for a specified period of time. This ability to control many of the variables affecting a terrestrial mammalian system was probably instrumental in increasing the sensitivity of the assays we used in this study. Furthermore, site specific toxic effects were able to be delineated between reference and treatment mesocosms. As a result, mesocosms have proved to be effective tools for monitoring site-specific, physiological responses of feral rodents to contaminant exposure.

In our trials, equal numbers of juvenile male and female cotton rats were introduced to each mesocosm and provided with food and water ad libitum over the duration of exposure. At the end of the exposure period, animals were collected for necropsy and physiological response assessed.

### Immunotoxicity Assessment

Our laboratory utilized a battery of assays to profile immunocompetence of animals. The controlled in situ environments provided by the mesocosms enabled us to conduct a variety of in vivo assessments of humoral and cell-mediated immunity, as animals could be periodically recaptured and handled as needed. Immune organ size and cellularity, basic hematological screening, and general health screens were performed on all individuals returned to the laboratory. Additionally, in vitro assays of immune system function included lymphoproliferative mitogenic responsiveness, lymphocyte subtyping, natural killer cell tumoricidal activity, and macrophage metabolic and phagocytic activity. In vivo assays included 24-hr phytohemagglutinin (PHA) hypersensitivity and delayed-type hypersensitivity to a recall-antigen (oxazolone) to assess cell-mediated immune function, and keyhole limpet hemocyanin challenge (T-dependent protein antigen) was used to assess humoral immunity.



One of the most revealing indicators of immunotoxicity was the in vivo assessment of cell-mediated immune function. Hypersensitivity responses of animals to PHA (Fig. 15) on the three reference sites were approximately 60% greater than those housed on suspected sites of toxicity. Interestingly, the reverse trend was observed for the delayed-type hypersensitivity reaction to the recall-antigen oxazalone (Fig. 16). Although both assays measure in vivo cell-mediated immune function, they differ greatly in their mode of action. The PHA assay was a measure of the degree to which the animal could respond to a known mitogenic substance where memory cells are not involved. In comparison, the oxazalone reaction measures memory cell function as well. An important component of the immune system is the self regulatory mechanism, whereby cells modulate or down-regulate immune response. The observed in vivo cell-mediated immune responses have lead us to hypothesize that the mixture of petrochemical contaminants at the study site have induced a lesion within the suppressive branch of the immune system. It is important to remember that any deviation from the normal response level, enhancement or reduction, can detrimentally affect survival of the host animal.

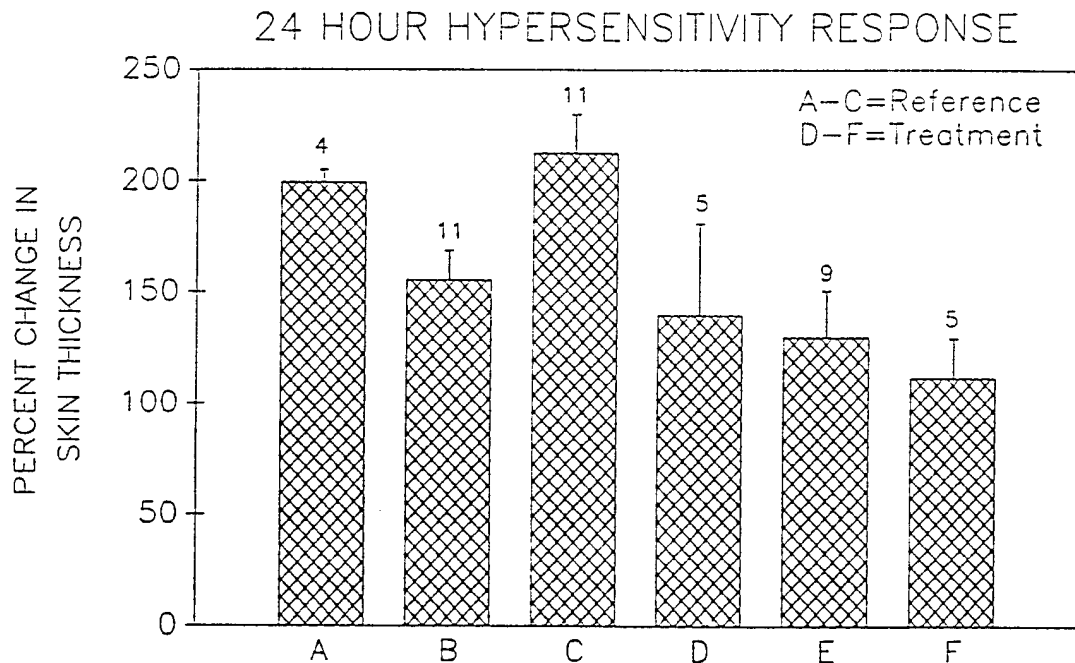


Fig. 15. Differences in in vivo cell-mediated immunity as measured by the 24-hr hypersensitivity response to an intradermal injection of phytohemagglutinin in cotton rats housed in mesocosms. Response was measured as percent change in skin-fold thickness of animals returned from mesocosms located on reference (A-C) and toxic (D-F) sites.

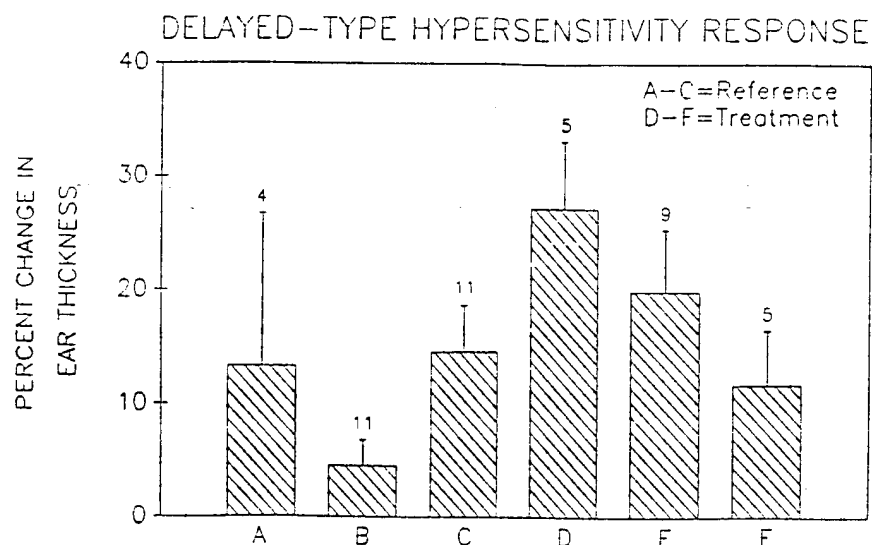


Fig. 16. Differences in in vivo cell-mediated immunity as measured by the delayed-type hypersensitivity response to a percutaneous application of oxazolone to previously sensitized cotton rats housed in mesocosms. Response was measured as percent change in ear thickness of animals returned from mesocosms located on reference (A-C) and toxic (D-F) sites.

### Metabolic Indicators of Toxicity

Animals housed in mesocosms on the contaminated and reference areas were also analyzed for cytochrome P450 induction using metabolism of alkoxyresorufin ethers, which are metabolized by the cytochrome P450 system. In all mesocosm studies, methoxyresorufin, ethoxyresorufin, propoxyresorufin, and pentoxyresorufin activity results were highly variable from sample to sample and no significant differences were observed. Given the fact that we observed evidence of immunotoxicity in cotton rats housed in mesocosms, two plausible explanations for the lack of effects on cytochrome P-450 activity are offered. It may be that the mixture of contaminants that existed on mesocosm sites are specific in their effects on the immune system, with no apparent effects on cytochrome activity. Another possibility is that at the concentrations of contaminants at these sites, immune cells are just more sensitive than the cytochrome P-450 system.

### Laboratory Sensitivity Assays

In order to adequately address the utility of wildlife species as environmental bioindicators, controlled laboratory studies are required to assess their sensitivity to selected environmental contaminants. Our principal objective in these series of experiments was to assess the sensitivity of various biomarkers used in our multiparameter model of wild cotton rats (*Sigmodon hispidus*) to the acute and chronic effects of exposure to benzene, inorganic lead, and combinations of stressors. In addition, we were interested in documenting the effects of specific contaminants on general morphological and reproductive condition of animals. We hypothesized that response parameters of cotton rats would be sensitive indicators of contaminant exposure.

### Lead Toxicity Trials

Lead is typically associated with effects on the central nervous, hematopoietic and renal systems (Goyer and Rhyne 1973), most notably disruption of porphyrin synthesis. This effect is associated with decreases in heme containing constituents including the hepatic microsomal hemoprotein cytochrome P-450. Lesions of lead toxicity in rats also include dullness of hair, basophilic stippling of the red cells, large deposits of iron in the spleen, hard and brittle long

bones. Renal lesions with irregularity of the tubular epithelium, hypertrophy of the nuclei and intranuclear eosinophilic acid fast inclusion bodies have been well documented (Finner and Calvery 1939). Acid fast intranuclear inclusions (lead inclusions) within renal and hepatic cells are considered virtually pathognomonic for lead poisoning (Zook 1972). Although the data base on the toxic properties of lead is growing, most studies have focussed on laboratory animals and humans. In order to adequately address the utility of wildlife species as environmental indicators of lead toxicity, we examined the pathologic effects of acute and chronic exposure to inorganic lead and in addition to study the effects of lead on microsomal cytochrome P-450 in cotton rats

We examined the effects of lead exposure on cellular immunity, hematology, and reproductive and body condition in mature cotton rats consisting of two groups of 36 rats exposed to 0, 100, or 1000 ppm lead in drinking water for either 7 or 13 weeks. Specific and non-specific cell-mediated immunity was assessed by measuring splenocyte proliferative responses to polyclonal mitogens (Concanavalin A and Pokeweed mitogen), *in vivo* 24-hr delayed-type hypersensitivity, metabolic activity of peritoneal macrophages, spleen mass and cellularity, and immune organ development. General physiological condition was assessed from hematological, morphological, and reproductive measures. Thorough necropsy examinations were performed and recorded in all the cases; all tissues were routinely processed and histological sections of adrenals, bone (femur), brain, epididymis, heart, kidney, liver, lung, lymph node, muscle, ovary, spleen and testicle were examined (special stains included Ziehl-Neelsen's acid fast stain and Fite's ferric on all sections of liver and kidney).

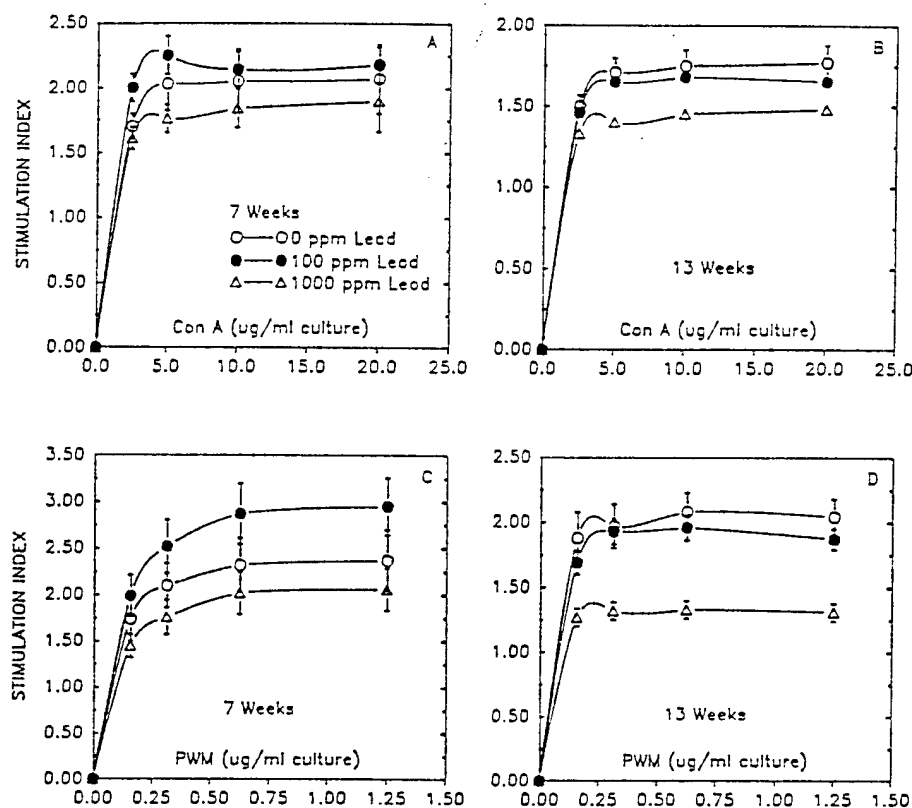


Fig. 17. Mean stimulation indices ( $\pm$ SE) of splenocyte proliferation following *in vitro* stimulation with four concentrations of Con A (2.5, 5, 10, and 20 ug/ml culture; A and B) and pokeweed mitogen (PWM; 0.156, 0.313, 0.625, and 1.25 ug/ml culture; C and D) in cotton rats consuming water containing 0, 100, or 1000 ppm lead acetate for 7 or 13 weeks.

Immune function was sensitive to lead exposure as indicated by depressed proliferative responses of cultured splenocytes, smaller popliteal lymph nodes, and larger spleens among lead-exposed cotton rats; thymus mass was suppressed at 1000ppm dose. A variety of hematological parameters also were sensitive to lead exposure as total leukocytes, lymphocytes, neutrophils, eosinophils, total splenocyte yield, packed cell volume, hemoglobin, and mean corpuscular hemoglobin were either negatively or positively impacted. Lead exposure altered proliferative responses of cultured splenocytes stimulated with varying dosages of PWM and Con A mitogen (Fig. 17). No differences in in vivo functional tests of cell-mediated immunity or macrophage function were noted.

The total cytochrome P-450 content between the controls and the treatment rats were not significantly different for both the exposure periods (7 and 13 weeks) and also between the 1000 ppm and 100 ppm treatment groups for both the exposure periods (Table 8). However the results of the phenoxazone assays revealed a significant difference in the P-450 activity between the control and treatment rats (Fig. 18); O-dealkylation of resorufin ethers in treatment rats was higher than the control rats. Methoxyresorufin was the most actively metabolized substrate and the rate of its metabolism by lead-induced microsomes from rats receiving 100 and 1000 ppm of lead acetate for both 7 and 13 weeks was significantly different from the control values. However, the ethoxyresorufin O-demethylase activity was only significant ( $P < 0.05$ ) in rats on 1000 ppm of lead for 7 weeks over the controls. Interestingly, rats on 100 ppm of lead for 13 weeks (and not 7 weeks) had significantly ( $P < 0.05$ ) higher rates of demethylase activity over rats on 1000 ppm of lead acetate. The activity difference of the benzyloxyresorufin in control and treatment rats was minimal.

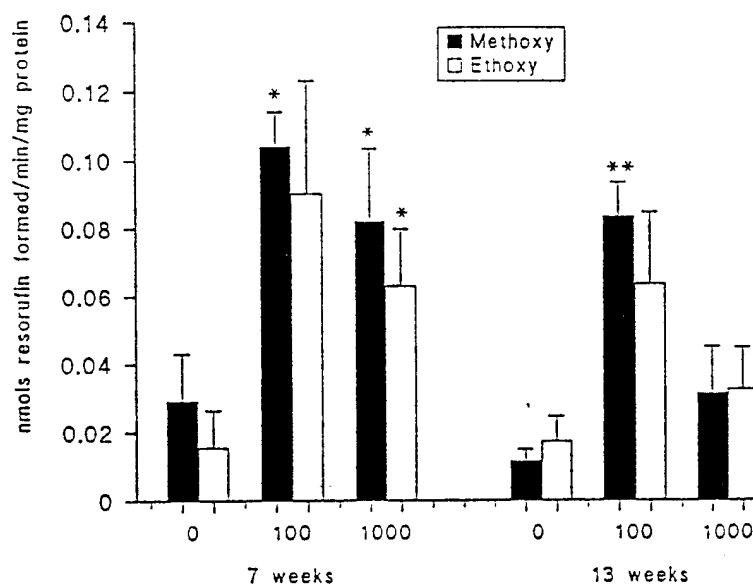


Fig. 18. Effect of lead treatment for 7 and 13 weeks on the microsomal O-dealkylation of methoxy and ethoxyresorufins in cotton rats. Values are mean of 6 rats  $\pm$  S.D. \*Mean significantly different from control group mean,  $P < 0.05$ . \*\*Mean significantly different from control and 1000 ppm group mean,  $P < 0.05$ .

Table 8. Liver weights and levels of hepatic cytochrome P-450 (Mean  $\pm$  S.E.) in cotton rats exposed to 0, 100, or 1000 ppm lead in drinking water for either 7 or 13 weeks.

	7 Weeks			13 Weeks		
	0 ppm	100 ppm	1000 ppm	0 ppm	100 ppm	1000 ppm
Liver weight ( % g B.W.) ( mean of 12 rats )	3.6 (0.4)	2.8 (0.2)	2.7 (0.3)	2.9 (0.3)	(0.3)	2.5 (0.2)
Cytochrome P-450 nmoles/mg protein ( mean of 6 rats )	1.026 (0.16)	1.28 (0.13)	1.309 (0.07)	0.745 (0.14)	0.911 (0.14)	0.867 (0.13)

General condition and reproductive parameters influenced by lead exposure included depressed mass of liver, seminal vesicles, and epididymes in males following a 7-week exposure. In general, lesions were more pronounced with increasing levels of lead in drinking water and longer exposures. On light microscopic examination of H & E-stained sections, altered renal proximal tubular epithelium was found in all rats dosed with 1000 ppm of lead acetate for either 7 or 13 weeks. The changes in the tubules were quite uniform in all the specimens. Most epithelial cells were enlarged and had irregular apical borders encroaching upon the lumina of the tubules. Occasionally necrosis of tubular epithelium and sloughing of cells into the lumina of tubules was observed. Intra-nuclear inclusions were present in the kidneys of all the 24 rats receiving 1000 ppm of lead acetate for either 7 or 13 weeks; however, inclusions were not present in the liver. The inclusions were usually solitary, spherical and varied from 2 to 7 microns in diameter and were pink to red in H & E stained sections and were usually found in enlarged nuclei with margined chromatin. Most inclusions were in the straight segments of proximal tubules. The inclusions were not acid fast with Ziehl-Neelsen's method which is routinely used to demonstrate lead inclusions. However the inclusions were acid fast with Fite's ferraco method. The glomeruli in these kidneys were normal. Rats which received 100 ppm of lead acetate for 7 or 13 weeks had the tubular changes but failed to demonstrate the lead inclusions in the kidney or liver. Sections of the kidney from the control animals did not contain the inclusions and were entirely free from the tubular changes. The presence of inclusion bodies reflect the amount of lead that can be found in body tissues (Zook 1972). Intranuclear inclusions in experimentally lead poisoned mice have been shown to contain lead as well as an uncharacterized protein. It was suggested that the inclusions represent a means by which the cell can safely store lead away from the mitochondria which are readily damaged by lead (Goyer et al. 1970).

Reproductive systems, especially in males, were highly sensitive to lead exposure. Development of accessory sex glands and epididymes were delayed or suppressed in rats dosed with lead. Testicles of 3 rats on 1000 ppm of lead for 13 weeks had fewer spermatozoa and in one rat ( 7 weeks) there was virtually no spermatogenesis. Three rats on 100 ppm of lead for 13 weeks had fewer sperm with no sperm in one rat. Ovaries of 4 rats on 1000 ppm of lead ( 3 of 13 week and 1 of 7 week ) consisted of corpora lutea with few developing follicles. Only 2 rats ( 7 week ) receiving 100 ppm of lead had the ovarian lesion. Testicles and ovaries from the control rats were normal.

Results in this study indicated that cellular immune responses, reproduction, and general hematology were sensitive to lead exposure. As might be expected, lesions were more severe with increasing levels of lead and longer exposure. Mean expectation of further life for cotton rats in wild populations ranges from approximately 8 weeks to 14 weeks. Therefore, cotton rats

inhabiting contaminated sites would potentially be exposed to lead for periods of time shown to impact physiological integrity.

### Benzene Toxicity Trials

Cotton rat sensitivities to varying concentrations of benzene and cyclophosphamide (a known immunosuppressant) were investigated in young and old animals in a series of three experimental trials. Additionally, we investigated their responses to simultaneous exposures to both benzene and protein nutritional stress to replicate the natural environmental conditions that exist in the field during late summer.

**Adult Trial.**--In trial 1, adult cotton rats were randomly assigned to 1 of 6 experimental treatments, with 12 animals per treatment. Treatments included 4 benzene (Aldrich Chemicals, Milwaukee, WI) dosages (100, 300, 600, or 1000 mg/kg body weight), cyclophosphamide (CY, Sigma Chem. Co., St. Louis, MO) as a positive control (50 mg/kg body weight), and corn oil as a negative control. All treatments were administered intraperitoneally (IP) for 3 consecutive days. Benzene was administered in corn oil on days 1-3 of the experimental period. Positive and negative controls received corn oil on days 1-3 at volumes equal to benzene doses. Cyclophosphamide (CY) in distilled water was administered to positive control animals on days 8-10. On day 7, all cotton rats received a 0.2 ml IP injection of a 10% sheep red blood cell (SRBC, Colorado Serum Co., Denver, CO) suspension in PBS. The trial was terminated and necropsies performed on day 12.

Thymus weights were markedly depressed among CY-treated cotton rats compared to benzene-treated ( $P < 0.003$ ) and negative control ( $P < 0.02$ ) groups. A dose response ( $P < 0.006$ ) in spleen weights was indicated among benzene treatments, with greater spleen weights among cotton rats receiving 1000 mg/kg than 100 mg/kg. Cyclophosphamide-treated animals also had significantly ( $P < 0.05$ ) lower spleen weights than the 1000 mg/kg benzene group. Hematocrits, WBC counts and viable splenocyte yields were significantly ( $P < 0.05$ ) influenced by experimental treatments. WBC counts (cells  $\times 10^3/\text{mm}^3$ ) were greatest for cotton rats receiving 600 mg/kg benzene ( $\bar{x} \pm \text{SE} = 7.99 \pm 1.37$ ) and lowest for CY-treated animals ( $3.45 \pm 0.55$ ). Total viable splenocyte yields (cells  $\times 10^6$ ) were greatest among animals receiving 1000 mg/kg benzene ( $151.5 \pm 19.1$ ) and lowest among those exposed to CY ( $70.1 \pm 8.6$ ). Cotton rats treated with CY had significantly ( $P < 0.001$ ) lower lymphocyte counts than benzene treatments and negative controls.

Sensitivity of indices of immunotoxicity were low. Cotton rats receiving either 300 mg/kg or 1000 mg/kg benzene had more SRBC-responding plaque forming cells per spleen (PFC/spleen) ( $P < 0.05$ ,  $P < 0.03$ ) and PFC/mg spleen ( $P < 0.05$ ,  $P < 0.06$ , respectively) than those in the 100 mg/kg treatment group. Specific contrasts indicated that both PFC/spleen ( $P < 0.04$ ) and PFC/mg spleen ( $P < 0.07$ ) were greater for benzene-treated than CY-treated cotton rats. Differences between negative control animals and other treatment groups were not significant ( $P > 0.10$ ). No significant differences ( $P > 0.10$ ) among treatments were observed in lymphoproliferative responsiveness to either Con A or PWM. Mean absorbances of nonstimulated cultures were significantly greater among CY-treated cotton rats than for benzene-treated ( $P < 0.05$ ) or negative control ( $P < 0.02$ ) animals. Cellular immunity measured as immune responsiveness to a topically applied antigen showed no significant ( $P > 0.10$ ) differences among experimental treatments.

Although some apparent dose related responses to benzene were seen (PFC/mg spleen, PFC/spleen, spleen weight), acute benzene exposure induced neither immunosuppression or stimulation in cotton rats, given the exposure regime used in our study. Our data strongly suggests that metabolism and excretion of metabolites in the cotton rat were probably complete by 9 days after benzene exposure. Cyclophosphamide, like benzene, is quickly metabolized and targets mitotic cells and its effectiveness as an immunosuppressant is lost within a few days after challenge. The lack of benzene-induced immunosuppression in this study suggested that cotton rats have efficient biotransformation capabilities and can apparently recuperate quickly from

leukopenia, thus highlighting necessary precautions when using *in situ* biomonitors of immunotoxicity. Animals caught on contaminated sites and held in the laboratory during acclimation and/or immunization periods may eliminate considerable amounts of xenobiotics and recover from abnormalities caused by the contaminants.

**Juvenile Trial.**--A total of forty 21-day old weanling (weaned at 16-18 days of age, all conceived in the wild and born in our laboratory animal facility) cotton rats were exposed to 2 benzene dosages (100 or 1000 mg/kg body weight), cyclophosphamide as a positive control (50 mg/kg body weight), and corn oil vehicle as a negative control, administered intraperitoneally for 3 consecutive days, similar to the adult trial above. We observed no gross behavioral changes; neuromuscular abnormalities or mortality during the trial in any of the cotton rats receiving benzene. One animal from the 1000 mg/kg benzene group died during the experiment from non-treatment related causes.

Weanling cotton rats exposed to CY ( $P < 0.001$ ) or benzene ( $P < 0.032$ ) had significantly depressed thymus weights compared to negative controls. Conversely, liver and kidney weights were greater among CY-treated animals compared to negative controls ( $P < 0.009$ ,  $P < 0.029$ ), respectively. WBC counts, hematocrits, and viable splenocyte yields were significantly ( $P < 0.001$ ) influenced by experimental treatments with significant depression induced by CY compared to benzene and negative control groups. Cotton rats treated with CY had significantly lower lymphocyte ( $P < 0.001$ ) and neutrophil ( $P < 0.019$ ) counts than negative control animals. Cellular immunity as measured by delayed-type hypersensitivity response to a percutaneously applied antigen, was significantly ( $P < 0.076$ ) influenced by experimental treatment as well. Specific contrasts showed a significant ( $P < 0.036$ ) depression in DTH responsiveness of cotton rats exposed to CY ( $5.53 \pm 1.9\%$ ) but not benzene ( $12.18 \pm 3.3$  and  $17.21 \pm 5.7$ ) compared to negative controls ( $15.78 \pm 3.9$ ).

It was clear from these trial that immunocompetence of juvenile animals is not directly comparable to adult rodents. Although cellular and humoral components necessary for a mature immune response may be present in early juvenile life, overriding humoral and/or cellular suppressive factors and inefficient cellular interactions may interfere with a normal response. In addition, differences in level and function of the enzymes responsible for detoxification in adults and juveniles complicate comparisons of their responses to toxicants.

**Multiple Stressor Trial.**--This study was designed to explore the effects of acute xenobiotic exposure and severe protein restriction on selected measures of cell-mediated immunity in juvenile cotton rats. In addition, possible synergistic or antagonistic influences of protein restriction on contaminant-induced immune modulation were explored. Our laboratory has demonstrated that individually, both protein restriction and benzene exposure have been shown to influence immunity in cotton rats.

A total of 41 (47-day old juvenile cotton rats) were weaned at 18 days post partum and at 21 days of age, litters were split and maintained on one of two isocaloric diets containing either 4% or 16% crude protein (United States Biochemical Corp., Cleveland, OH 44128) for a 26 day experimental period. Litters were further divided among 5 toxicant treatments resulting in a 2 X 5 factorial design. Experimental treatments included 3 benzene dosages (100, 500, or 1000 mg/kg body weight), cyclophosphamide as a positive control (50 mg/kg body weight), and corn oil vehicle as a negative control, administered intraperitoneally (IP) for 3 consecutive days.

Twenty six days of dietary crude protein restriction had a significant impact on most measures of immunocompetence. Relative weights of spleen, thymus, and liver, as well as neutrophil and eosinophil counts and splenic cellularity, were significantly depressed in animals on the 4% crude protein diet. In contrast, delayed-type hypersensitivity was enhanced in the protein-restricted group. Enhanced DTH response is consistent with findings of other studies dealing with protein restriction in cotton rats. The 4% protein diets in this study represent a severe crude protein restriction as indicated by the lack of growth observed in the protein restricted group; cotton rats tend to select natural food items (primarily monocots) with low crude protein content.

We expected that synergistic and/or antagonistic interactions between diet level and toxicant exposure would be evident in our study. Relative spleen weight resulted in the only indication of a significant interaction between diet restriction and contaminant exposure. It appeared that some degree of splenic hypertrophy resulted from benzene toxicity in cotton rats on the 4% protein diet which was not evident in the 16% protein group. No other statistically significant interactions were observed due possibly to the severity of the dietary restriction. Cotton rats maintained on 4% protein diets may have been immunosuppressed to the point that additional immunotoxic insults were not measurable or biologically significant. Additionally, severe protein restriction is known to impair metabolic processes often required to produce toxic metabolites from many xenobiotic compounds.

### Aroclor 1254 Trials

Aroclor 1254, a mixture of polychlorinated biphenyls, is a xenobiotic of concern to toxicologists because of its widespread distribution in both terrestrial and aquatic food chains, and its potential toxicity effects in terrestrial ecosystems. It is known to induce hepatic microsomal *O*-dealkylation of ethoxyresorufin and pentoxyresorufin in *in vitro* studies in laboratory rats, however, its effects on the microsomal *O*-dealkylation of wild rodents had not previously been studied. In this study male laboratory raised cotton rats (4 per dose group) were dosed with a single IP injection of 0, 15, 60 or 250 mg/Kg of Aroclor 1254 in corn oil. They were sacrificed 4 days later for evaluation of hepatic cytochrome metabolic function. *In vitro* alkoxyresorufin metabolism of microsomes was performed as previously described using ethoxyresorufin, pentoxyresorufin and propoxyresorufin as substrates (Elangham et al. 1991).

Aroclor 1254 at all doses resulted in significant ( $P < 0.005$ ) induction of metabolism of ethoxyresorufin, pentoxyresorufin, and propoxyresorufin (Fig. 19); similar levels of induction were seen at all dosage levels. We observed that total hepatic cytochrome activity was increased ( $P > 0.01$ ) at all dosage levels of Aroclor 1254 (Fig. 20). Aroclor 1254 resulted in induction of both ethoxyresorufin and pentoxyresorufin. We have previously shown in the cotton rat that the *O*-dealkylation of ethoxyresorufin was preferentially induced by the prototype inducer 3-methylcholanthrene and that the *O*-dealkylation of pentoxyresorufin was preferentially induced by the prototype inducer phenobarbital. These results are similar to those observed in common laboratory rat strains (Burke et al. 1985, Novak and Qualls 1989). In this study Aroclor 1254 caused a mixed type of induction to both ethoxyresorufin and pentoxyresorufin in the cotton rat (Fig. 21). Similar findings have been observed in common laboratory rat strains (Lubet et al. 1990). The degree of induction was similar at all dose levels showing that in the cotton rat, alkoxyresorufin metabolism is easily induced by Aroclor 1254.

Aroclor 1254 is also known to be a potent immunotoxicant, which we explored by measuring immune responsiveness relative to untreated controls and cyclophosphamide-immunosuppressed controls. Aroclor 1254 has little effect on spleen size, splenic cellularity, and hematology, whereas, cyclophosphamide significantly ( $P < 0.03$ ) WBC counts. The primary immunological effects were observed in lymphoproliferative responsiveness of cultured splenocytes stimulated with the mitogen ConA (primarily stimulates T cells). Lymphoproliferative responses to 5 and 10  $\mu\text{g/ml}$  culture of ConA were significantly greater among Aroclor-treated cotton rats compared to untreated controls ( $P < 0.01$ ). There was also a tendency ( $P < 0.07$ ) for greater responses among untreated controls than Aroclor-treated rats stimulated with the T and B cell mitogen PWM (pokeweed mitogen).

These trials indicate that cotton rats are sensitive to the effects of polychlorinated biphenyls, and thus, should be a reliable bioindicator organism for indexing the occurrence and severity of such contaminants in terrestrial ecosystems.



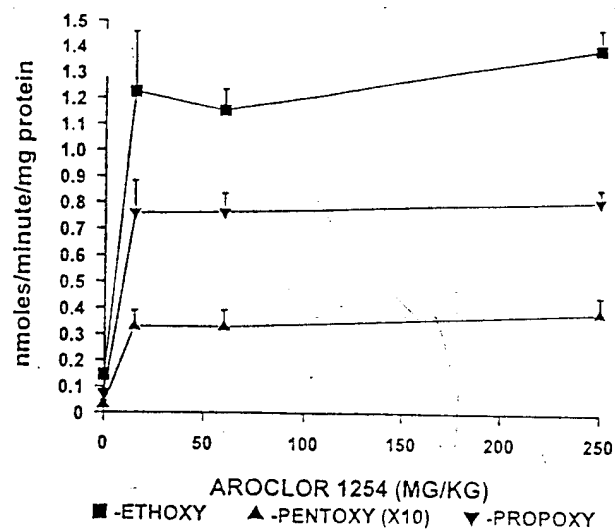


Fig. 19. Hepatic microsomal alkoxyresorufin metabolism in the cotton rat after receiving Aroclor 1254. The level of pentoxyresorufin is expanded 10-fold to facilitate visualization. All levels of all ethers in groups receiving Aroclor are significantly increased when compared to controls ( $P < 0.005$ ).

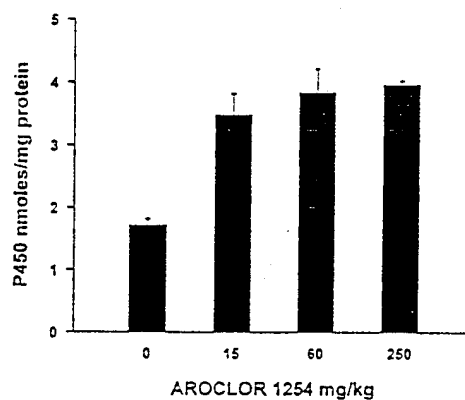


Fig. 20. Total hepatic cytochrome P450 in cotton rats 4-days after receiving Aroclor 1254.

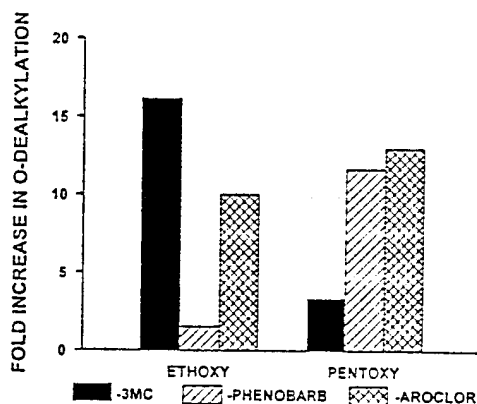


Fig. 21. Comparison of ethoxyresorufin and pentoxyresorufin metabolism from cotton rats exposed to 3-methylcholanthrene, phenobarbital, and Aroclor 1254. The fold increase represents the induced level divided by appropriate control.

### Benzo-a-pyrene (BAP)

This experiment was conducted in laboratory raised male cotton rats. Briefly 2 cotton rats were randomly assigned by body weight to 5 dose groups of BAP with an average body weight of 160.19 g between the groups. Five logarithmic doses of BAP viz. 0, 3, 10, 30, and 70 mg/kg were used. BAP was suspended in filtered corn oil and the rats were injected intra-peritoneally for 4 days. Control rats received an identical volume (2 ml) of corn oil. Rats were killed 24 hours after the last dose by CO<sub>2</sub> inhalation. At sacrifice whole body and liver weights were obtained. At least 2 grams of the liver was collected in chilled KCl for preparing microsomes. The remaining liver was collected in neutral buffered formalin for histopathologic examination. Hepatic microsomes were prepared using standard techniques. The formalin fixed liver was routinely processed and stained with haematoxylin and eosin for light microscopic examination. The cytochrome P-450 dependent enzyme activity was measured by the O-dealkylation of resorufin ethers. The dealkylation of alkoxyresorufins (propoxy, pentoxy, methoxy, ethoxy and benzyloxyresorufin) was measured at 37° C using a spectrofluorometer (Fluoromax) using previously described methods.

Microsomal dealkylation of resorufin ethers in cotton rats treated with BAP for 4 days responded in a dose-response fashion, as expected (Table 9). Induction of enzymes were dramatic among those in the 70mg/kg treatment group (Fig. 22). Cotton rats receiving 30 and 70 mg/kg BAP had hepatocellular hypertrophy compatible with cytochrome P450 induction.

Table 9. Values are mean of 2 rats expressed as nmoles of resorufin formed /min/mg microsomal protein. Fold induction over the control rats with corresponding ethers is given in parenthesis.

	Propoxy	Pentoxy	Ethoxy	Methoxy	Benzyloxy
0 mg	0.05	0.01	0.04	0.06	0.02
3	0.12 (2)	0.01 (0)	0.12 (3)	0.14 (2)	0.03 (2)
10	0.10 (2)	0.02 (2)	0.11 (3)	0.20 (3)	0.04 (2)
30	0.12 (2)	0.02 (2)	0.19 (5)	0.30 (5)	0.04 (2)
70	0.31 (6)	0.19 (19)	0.34 (9)	0.72 (12)	0.13 (7)

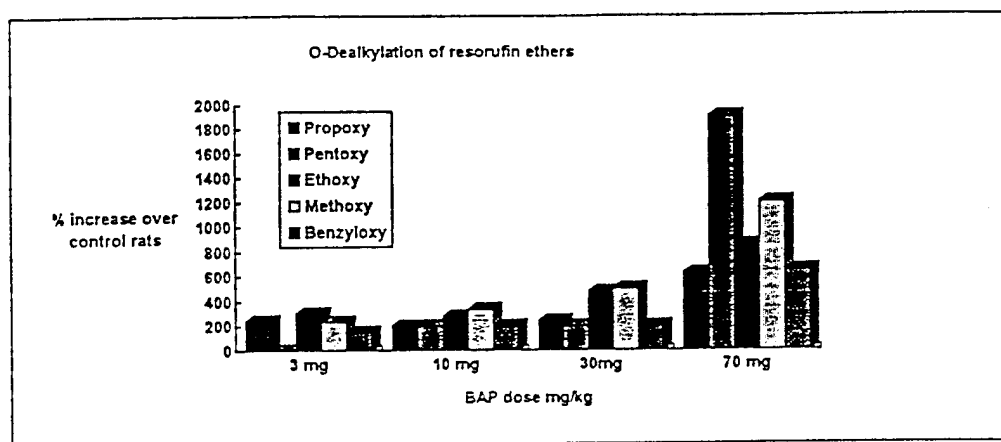


Fig. 22. Percentage increase in the microsomal O-dealkylation of resorufins in BAP-treated cotton rats compared to those receiving corn oil (controls).

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- Bowers, B., M. Thies, and K. McBee. Radiation and chemical-induced chromosomal damage in wild cotton rats. Southwestern Association of Naturalists, Southwest Missouri State University Springfield, NO. 15-17 April 1993.
- Bowers, B., M. Thies, and K. McBee. Radiation and chemical-induced chromosomal damage in wild cotton rats. American Society of Mammalogists. Western Washington University, Bellingham, WA 19-23 June 1993.
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- Burks, S. L., E. Stebler, and A. Sampley. 1992. Workshop on use of Microtox NOEC for toxicity reduction evaluations of wastewater samples. Paper presented at S. Central Soc. Environ. Toxicol. Chem., May 1992, Houston, TX.
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